The use of Toll-like receptor ligands as adjuvants in fish vaccines

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by

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Paper II:

Structurally diverse genes encode Tlr2 in rainbow trout: The conserved receptor cannot be stimulated by classical ligands to activate NF-kappaB in vitro.

Brietzke A, Arnemo M, Gjøen T, Rebl H, Korytář T, Goldammer T, Rebl A, Seyfert HM. *Developmental and Comparative Immunology (2016). 54, 75-88.* doi:10.1016/j.dci.2015.08.012

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Kavaliauskis A, Arnemo M, Kim SH, Ulanova L, Speth M, Novoa B, Dios S, Evensen Ø, Griffiths GW, Gjøen T. *Zebrafish (2015). 12, 421-431.* doi:10.1089/zeb.2015.1126

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Summary of thesis

Non-living antigens are often poorly immunogenic and require addition of adjuvants to elicit protective immunity. Due to the immunostimulatory potential of Toll-like receptor (TLR) ligands, they are explored as vaccine adjuvants. The development of efficient and cheap vaccines against aquatic viruses is important for a sustainable aquaculture industry and the adjuvants for fish vaccines need to be improved. However, increased knowledge of fish TLR function is required before their ligands can find their way into fish vaccines.

The major aim if this thesis has been to contribute to a more detailed understanding of fish TLRs. First, the tissue distribution of all known Atlantic salmon TLRs, the immunostimulatory potential of a panel of TLR ligands in primary head kidney leucocytes, and the impact of viral infection on TLR expression in head kidney were investigated. Head kidney and spleen were the main TLR expressing organs in Atlantic salmon. Several TLR ligands induced expression of inflammatory cytokines in salmon head kidney leucocytes. TLR3, TLR7, and TLR8a1 were induced *in vivo* after viral infection. In order to functionally validate ligand-specific activation of fish TLRs, we established an *in vitro* reporter assay in a salmon cell line. However, classical TLR2 ligands failed to activate rainbow trout TLR2 signalling when using NF-κB activation as measure of activation. To test the *in vivo* immunostimulatory potential of a TLR ligand alone and in vaccine formulations, a cold-water zebrafish challenge model was used. The TLR3 ligand poly I:C induced expression of antiviral transcripts in zebrafish head kidney and pre-treatment with poly I:C delayed VHSV (viral haemorrhagic septicaemia virus)-induced mortality. Chitosan encapsulated poly I:C was demonstrated to provide protection against VHSV when co-injected with two different non-living antigens (inactivated whole VHSV and VHSV glycoprotein G). Due to decreasing levels of the dietary n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in Atlantic salmon feed, we investigated how minimal levels of these fatty acids affect TLR signalling in Atlantic salmon leucocytes. The ability of leucocytes to respond to TLR ligand stimuli was reduced with low dietary- and head kidney levels of EPA and DHA, indicating the importance of n-3 fatty acids in resistance to infection and response to vaccines.

Our results provide new knowledge in the fish TLR field and lend support to poly I:C as a promising adjuvant candidate in viral vaccines.

Sammendrag (Summary in Norwegian)

Adjuvanser er en gruppe substanser som ofte brukes for å øke immunogenisiteten til vaksineantigener uten egen replikasjon (inaktiverte virus og rekombinante proteiner). Ligander som gjenkjennes av Toll-lignende reseptorer (TLR) stimulerer immunstemet og utforskes som potensielle nye adjuvanser i humane vaksiner. Det er behov for bedre virusvaksiner og nye adjuvanser til bruk i den globale akvakulturnæringen. For å kunne fullt utnytte den stadig økende informasjon om TLR og TLR-ligander i vaksiner til andre arter, må man øke kunnskapen om TLR i fisk. Hovedmålet med denne avhandlingen var derfor å bidra til økt forståelse om TLR i fisk. I artikkel 1, undersøkte vi distribusjonen av alle kjente TLR i atlantisk laks, hvordan hodenyre leukocytter responderte på stimulering med TLR-ligander og hvordan genuttrykket av TLR ble endret under en virusinfeksjon. Hodenyre og milt var de organene som uttrykte høyeste nivåer av de fleste TLR, og flere av TLR-ligandene induserte økt uttrykk av inflammatoriske cytokiner i leukocytter fra hodenyre. TLR3, TLR7 og TLR8a1 ble oppregulert ved virussykdommen infeksiøs lakseanemi i laks. For å kunne måle ligandbinding til TLR fra fisk (artikkel 2), etablert et cellesystem basert på målinger av NF-κB-aktivitet (et nedstrøms signalprotein). TLR2 fra regnbueørret lot seg ikke aktivere med klassiske TLR2-ligander i dette systemet. Sebrafisk ble også brukt for å teste immunstimulerende effekt av en TLR-ligand og til utprøving av vaksiner basert på TLR-ligand formulert i nanopartikler (artikkel 3 og 4). TLR3-liganden poly I:C økte uttrykket av flere immungener i sebrafisk som er viktige i bekjempelsen av virussykdommer, samt at løselig poly I:C forsinket dødeligheten etter at fiskene var infisert med VHS (viral hemoragisk septikemi)-virus. Vaksiner som inneholdt poly I:C innkapslet i chitosan partikler kombinert med enten et inaktivert VHS-virus eller glykoprotein fra VHS-virus beskyttet sebrafisken fra VHS. Dette viser at poly I:C er en lovende adjuvans i virusvaksiner. På grunn av økt etterspursel etter eicosapentaensyre (EPA) og docosahexaensyre (DHA), har innholdet av disse omega-3 fettsyrene i laksefôr blitt kraftig redusert. Vi undersøkte derfor hvordan minimale nivåer av disse fettsyrene påvirker leukocytter fra atlantisk laks. Leukocytter fra gruppen som ble fôret med de laveste nivåene av EPA og DHA viste redusert evne til å respondere på TLR-ligander. Dette indikerer viktigheten av omega-3 fettsyrer for å bekjempe infeksjoner og evnen til å respondere optimalt på vaksiner.

Abbreviations

AP1	Activator protein 1
CLR	C-type lectin-like receptors
COX	Cyclooxygenase
CREB	Cyclic AMP-responsive element-binding protein
DHA	Docosahexaenoic acid
dsRNA	double stranded RNA
EPA	Eicosapentaenoic acid
FCA	Freund's complete adjuvants
FIA	Freund's incomplete adjuvants
GALT	Gut-associated lymphoid tissue
GSK	Glaxo Smith Kline
IFIT	Interferon-induced proteins with tetratricopeptide repeats
IFN	Interferon
IL	Interleukin
ILT	Interbranchial lymphoid tissue
IPN	infectious pancreatic necrosis
IPNV	Infectious pancreatic necrosis virus
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
ISA	infectious salmon anaemia
ISAV	Infectious salmon anaemia virus
ISG	IFN-stimulated gene
LBP	LPS-binding protein
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	leucine-rich region
LTA	Lipoteichoic acid
LTB4	Leukotriene B4
MAL	MyD88-adaptor-like
MAPK	Mitogen-activated protein kinases
MDA5	Melanoma differentiation-associated gene 5

MHC	major histocompatibility complex
MPL	3-O-desacyl-4'-monofosforyl lipid A
Myd88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear factor kappa B
NLR	Nucleotide oligomerization domain like receptors
OIE	The world organization for animal health
ODN	Oligodeoxynucleotides
PAMP	Pathogen associated molecular patterns
PD	Pancreas disease
PGE2	Prostaglandin E2
PGN	Peptide glycan
PGRP	Peptide glycan recognition protein
PLA2	Phospholipase A2
PLGA	Poly-(lactide-co-glycolide)
Poly I:C	Polyinosine–polycytidylic acid
PRR	Pathogen recognition receptor
RIG-1	Retinoic acid-inducible gene-1
RLR	Retinoic acid-inducible gene-1 like receptors
SARM	Sterile α-and armadillo-motif-containing protein
SAV	Salmon alphavirus
ssRNA	Single stranded RNA
Th1	Type 1 T helper cell
Th17	Type 17 T helper cell
Th2	Type 2 T helper cell
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-receptor-associated factors
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
VHS	Viral haemorrhagic septicaemia
VHSV	Viral haemorrhagic septicaemia virus
VLP	Virus like particle

Introduction

1. Vaccines

1.1. A brief history of vaccines

During the last century, vaccination has had a tremendous impact on global health by reducing death and morbidity caused by infectious diseases. Vaccines are biological preparations that enhance immunity against disease and either prevent (prophylactic vaccines) or treat disease (therapeutic vaccines) (Delany et al., 2014). The principle of vaccination was first applied over 1000 years ago via the process of "variolation"; the inoculation of pustules from smallpox patients into healthy individuals who were then subsequently protected against the disease (Riedel, 2005). The British vaccine pioneer Edward Jenner developed the process further in the 1790s by showing that exposure to cowpox induces protective immunity to smallpox (the term "vaccination" is derived from the Latin words for cow and cowpox - *vacca* and *vaccinia*). This discovery led to a decline in smallpox mortality and, many years later, the eradication of smallpox in 1977 (Riedel, 2005, Minor, 2015). Since then there have been major advances in vaccine development. Louis Pasteur's principles "isolate, inactivate and inject" in the late 1800s led to the development of successful vaccines, based on inactivated toxins and live attenuated or inactivated (killed) pathogens, against many serious infectious diseases (Rappuoli, 2007, Plotkin, 2005). From 1950, many new effective inactivated, live attenuated, and subunits vaccines have been developed as a result of the progress in microbiology and gene technology.

1.2. Human viral vaccines

Live attenuated vaccines against viral diseases are one of the most cost effective health interventions currently available (Bloom et al., 2005). Poliomyelitis, measles, mumps, yellow fever, and rubella are examples of diseases that can be prevented by licensed live attenuated vaccines (Minor, 2015). Poliomyelitis is nearly eradicated, and measles and mumps are controlled in the western world (Minor, 2015). Live vaccines are generally very effective and induce long-lived immunity after only one single dose. Attenuation can be achieved by serial passages of the virus in cultured cells, applying harsh condition on a

virus strain, or using recombinant gene technology (Rappuoli et al., 2009, Kallerup and Foged, 2015). The viral strain will accumulate mutations that make it non-pathogenic, while it still possesses patterns of the original virus and mimics the natural infection by inducing an immune response (Clem, 2011). Today, the further development of these vaccines is limited by several safety concerns, e.g. risk of reversion to the virulent strain, disease in immunocompromised individuals, and spread of the attenuated virus into the environment (Lauring et al., 2010).

The safety concerns of live attenuated vaccines have led to a shift towards inactivated viruses or viral subunits as vaccines. Inactivated vaccines are generally less immunogenic than their live attenuated counterparts due to the lack of replication and fast clearance from the injection site; hence the often need for an additional booster dose and adjuvants. However, such vaccines are more stable and safer than live vaccines. Inactivated vaccines are produced by viral cultivation to produce large quantities of the antigen and then inactivation by radiation, heat, or chemical agents. Inactivated vaccines usually do not require refrigeration, and they can be easily stored and transported in freeze-dried form, which makes them more accessible to people in developing countries (Sanders et al., 2015).

Subunit vaccines contain one or more components of a pathogen rather than the entire pathogen (like a protein, polysaccharide, glycoprotein, inactivated toxin, or outer membrane vesicle). The antigens are chosen because of their ability to elicit protective immunity. Production is more easily controlled and they offer considerable advantages over the inactivated and attenuated vaccines in terms of safety. Because of the lack of many pathogen features, these subunit vaccines are weakly immunogenic and often require co-administration of an adjuvant (Kallerup and Foged, 2015). Virus-like particles (VLPs) are derived from self-assembling subunits of viral structural proteins that mimic the structure of an authentic virus, but lack the viral genome. Vaccines consisting of VLPs combine the advantages of whole virus vaccines (strong immune response) and recombinant subunit vaccines (safe and simple vaccine). Several licensed VLP vaccines are available, e.g. against human papilloma virus and hepatitis B virus. These vaccines consist of one or more viral proteins (expressed in yeast, insect, or mammalian cells) that are important for inducing antibodies against the virus (Roldão et al., 2010).

DNA vaccines deliver genetic material that encodes a specific antigen to the skin or the muscle. The DNA enters local cells and uses the host cellular machinery to express the antigen encoded in the DNA vaccine. The synthesised antigen can then be presented in the major histocompatibility complex (MHC) for T and B cells to initiate immune responses. Two viral DNA vaccines are licensed (vaccines against West Nile virus and infectious hematopoietic necrosis virus for use in horses and salmon respectively), and several DNA vaccines are currently tested in clinical trials (Kutzler and Weiner, 2008). It is also possible to use replicating or non-replicating viruses as vaccine vectors. The virus vector is a non-pathogenic virus that has been modified to encode and present antigens from a pathogen. A wide range of innovative viral vectors that are able to deliver antigens and induce immune responses are available (e.g. pox viruses, adenovirus, coronavirus, flavivirus, influenza virus, rhabdovirus etc. (Draper and Heeney, 2010).

1.3. The need for viral vaccines in fish farming

Aquaculture (farming of aquatic organisms) is a rapidly growing global industry and an important nutritional and economical source for many countries around the world, especially in Asia and South America. The aquaculture industry is also of great importance to Norway. In the late 1960s, salmon farming started in Norway to support rural fishing communities that were having economic problems due to reduction in wild-capture fishing activity (Liu et al., 2011). Since then the aquaculture industry has overcome many technical and biological challenges and has become one of Norway's biggest export trades after oil and gas. The main species of Norwegian aquaculture today are salmonid fish (Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*). Today, Norway is the largest producer and exporter of Atlantic salmon globally, followed by Chile, United Kingdom, and Canada. In 2014, the total amount of produced Atlantic salmon in Norway exceeded 1.2 million tonnes, which constituted over 50 % of the total world production of this fish (Guttormsen, 2015).

Although aquaculture is one of the fastest growing food-producing industries in the world, there are still challenges that pose a threat to a sustainable growth of this industry. One of the major challenges is infectious diseases caused by bacteria, viruses, and parasites, whose detrimental impacts are facilitated by the effectiveness of pathogen transportation in water and the high density of fish in large-scale farming. Diseases can lead to great production losses, unacceptable animal welfare situations, and spread of disease to wild

fish in the area. Worldwide the most common pathogens causing infectious diseases are bacteria (over 50%), followed by viruses, parasites and fungi (Kibenge et al., 2012). Today, in Northern Europe and North America, bacterial diseases are controlled by vaccines in most salmonid farms and the use of antibiotics is limited (Sommerset et al., 2005). However, viral diseases have been more difficult to control due to lack of antiviral therapeutics, high susceptibility of fish during the early stages of the life cycle, and insufficient knowledge about pathogenesis and natural resistance to viral infections. The development of efficient viral vaccine has also been a challenge (Kibenge et al., 2012). Both farmed and wild fish are susceptible to a long list of viral pathogens. Some of the most important viral diseases and the causative viruses affecting farmed fish are listed in Table 1 (Kibenge et al., 2012, Crane and Hyatt, 2011, Dhar et al., 2014, Shoemaker et al., 2015). Eight viral fish diseases are listed as reportable diseases by the World Organization for Animal Health (OIE) in 2015 due to the risk of viral spread through commercial trade of fish and fish products (see Table 1) (Dhar et al., 2014). In Norway, pancreas disease (PD), heart and skeletal muscle inflammation (HSMB), infectious pancreatic necrosis (IPN), cardiomyopathy syndrome (CMS), and infectious salmon anaemia (ISA) are the most frequent viral diseases detected in farmed fish (Veterinærinstituttet, 2015).

Viral haemorrhagic septicaemia (VHS) is one of the oldest prevailing and most economically important viral diseases of salmonid fish in Europe and flounder in Asia. No vaccine is available, and the disease is reportable to OIE indicating the importance of this virus. The causative agent of VHS, viral haemorrhagic septicaemia virus (VHSV) is a single stranded RNA virus and member of the family *Rhabdoviridae* and genus *Novirhabdovirus*. The virus has five major structural proteins (nucleocapsid-, phospho-, matrix-, glyco- and RNA polymerase protein) and there are currently four genotypes identified (genotype I-IV) (Einer-Jensen et al., 2004, Skall et al., 2005). The most susceptible fish species is the rainbow trout, but the virus has since the first identification in the early 1900s been isolated from numerous wild and farmed fish species (Skall et al., 2005). VHSV causes high mortality rates (up to 100% in fry) and huge economical losses (Crane and Hyatt, 2011, Micol et al., 2005).

Table 1: List of viral diseases impacting farmed fish

Disease	Causative virus	Virus family	Host examples
Infectious pancreatic necrosis (IPN)*	Infectious pancreatic necrosis virus (IPNV)	*Birnaviridae*	Salmonids, halibut, common carp
Viral encephalopathy and retinopathy (VER) or Viral nervous necrosis (VNN)	Nervous necrosis virus (NNV)	*Nodaviridae*	Atlantic halibut, Atlantic cod, sea bass, grouper
Infectious salmon anaemia (ISA)*[a]	Infectious salmon anaemia virus (IAV)	*Orthomyxoviridae*	Salmonids
Pancreas disease (PD) or sleeping disease (SD)*[a]	Salmon alpha virus (SAV)	*Togaviridae*	Atlantic salmon, rainbow trout
Infectious hematopoietic necrosis (IHN)[a]	Infectious hematopoietic necrosis virus (IHNV)	*Rhabdoviridae*	Salmonids, sturgeon, herring
Epizootic hematopoietic necrosis (EHN)[a]	Epizootic hematopoietic necrosis virus (EHNV)	*Iridoviridae*	Rainbow trout, perch species
Viral haemorrhagic septicaemia (VHS)*[a]	Viral haemorrhagic septicaemia virus (VHSV)	*Rhabdoviridae*	Rainbow trout, turbot, flounder
Spring viremia of carp (SVC)[a]	Spring viremia of carp virus (SVCV)	*Rhabdoviridae*	Carp species
Cardiomyopathy syndrome (CMS)*	Piscine myocarditis virus (PMCV)	*Totiviridae*	Atlantic salmon
Heart and skeletal muscle inflammation (HSMI)*	Piscine reovirus (PRV) (suspected)	*Reoviridae*	Atlantic salmon
Koi herpesvirus disease (KHVD)[a]	Koi herpesvirus (KHV)	*Alloherpesviridae*	Common carp, Koi
Red sea bream iridoviral disease (RSID)[a]	Red sea bream iridovirus (RSIV)	*Iridoviridae*	Sea bream species

*Diseases reported in Norway.
[a] Listed as reportable fish diseases by The World Organisation for Animal Health (OIE).
References (Kibenge et al., 2012, Crane and Hyatt, 2011, Dhar et al., 2014, Shoemaker et al., 2015)

1.4. Current status of fish vaccines

The success of bacterial vaccines in fish have led to a decrease in the use of antibiotics, and several vaccines against bacterial diseases are used in aquaculture worldwide (Håstein et al., 2005). In Norway, bacterial diseases caused enormous losses to the salmon farming during the 1980s and tonnes of antibiotics were used (Figure 1). However, due to the introduction of effective bacterial vaccines and improved health management, the total consumption of antimicrobials was reduced by 99% and made it possible for the huge increase in production (NORM/NORM-VET2013, 2013). Today in Norway, the salmonid population is vaccinated against three major bacterial diseases (vibriosis, cold-water vibriosis, and furunculosis) before release into sea water. Worldwide vaccination has been most important in salmonids and species like sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) (Håstein et al., 2005). The main administration routes for fish are injection and immersion. Oral vaccines have also been used, but have not provided the same efficiency as immersion and injectable vaccines. The bacterial vaccines in use are simple, consisting of formalin-inactivated whole bacteria. Many of the registered vaccines

are multivalent, i.e. they protect fish against more than one bacterial disease. To obtain satisfactory protection, adjuvants are included in the vaccines (Håstein et al., 2005).

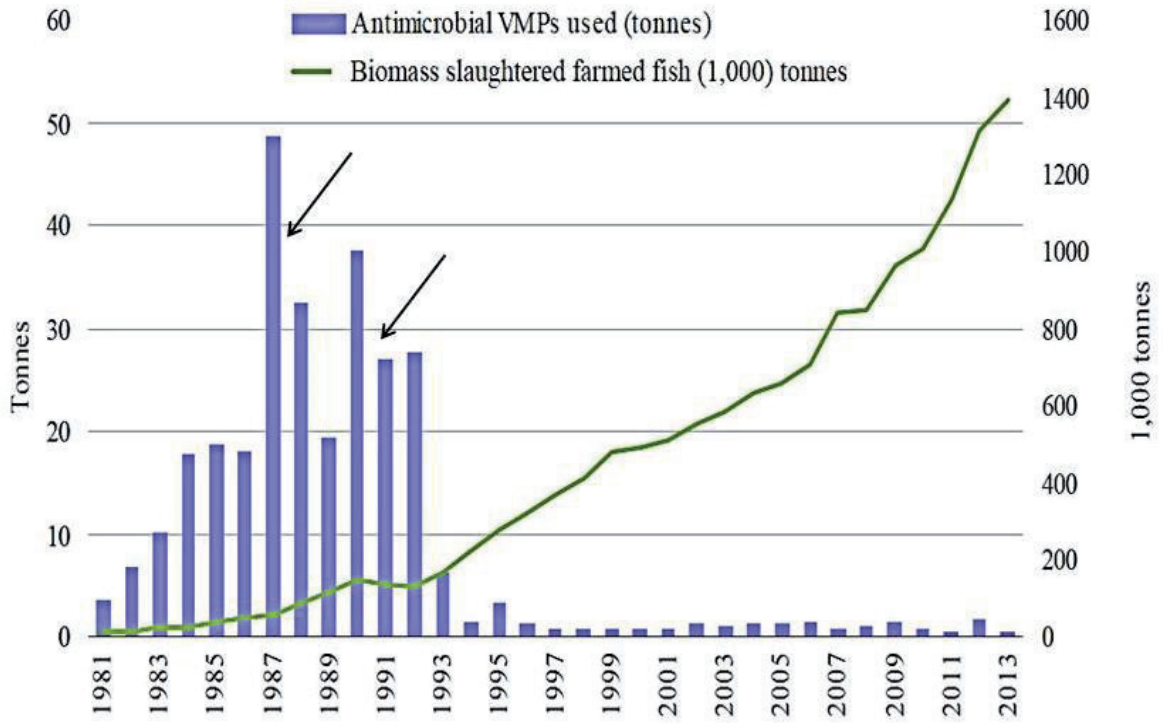

Figure 1: *Amount of antibiotics (tonnes) for therapeutic use in farmed fish in Norway versus produced biomass farmed fish (1,000 tonnes) (NORM/NORM-VET2013, 2013). The arrows mark the introduction of bacterial vaccines (Sommerset et al., 2005).*

Although vaccination against diseases in aquaculture has enabled control of many bacterial diseases, the development of efficient and cheap vaccines against viral diseases has proven very challenging. The few existing vaccines for viral diseases in fish are either monovalent vaccines or included in multivalent bacterial vaccines. Live attenuated vaccines induce strong and sustained immune responses to the target disease in fish, but there are environmental and regulatory concerns hampering their further development. Most aquaculture operations are without physical barriers to wild-living fish in the same area and strains attenuated for aquaculture species may be virulent in wild species living in farm areas (Dhar et al., 2014, Salgado-Miranda et al., 2013, Brudeseth et al., 2013).

The majority of the commercial viral vaccines are based on inactivated virus and target salmonid viruses like infectious pancreatic necrosis virus (IPNV), infectious salmon anaemia virus (ISAV), and salmon alphavirus (SAV) (Dhar et al., 2014). However, many

attempts to make inactivated viral vaccines (although adjuvanted) have failed due to low immunogenicity. In addition, some viruses display poor or non-existent replication in cell cultures, making it difficult to grow sufficient quantities of antigen for vaccine production. A few subunit vaccines are available against IPN and ISA, and consist of a capsid protein (VP2) of IPNV and the ISAV recombinant hemagglutinin esterase gene, respectively. One DNA vaccine, based on the gene of IHNV glycoprotein, is licensed in Canada, but is not approved in Europe or the United States due to safety concerns regarding the integration of foreign genes in food (Evensen and Leong, 2013). Although the licensed viral vaccines have been documented to be effective in experimental trials, the efficacy of these vaccines under field condition is uncertain due to a lack of published reports and continued occurrence of viral outbreaks (Rimstad, 2014, Robertsen, 2011, Kibenge et al., 2012, Rimstad, 2011, Veterinærinstituttet, 2015).

Like the inactivated bacterial vaccines, the inactivated virus and viral subunits are non-replicating antigens resulting in lower immunogenicity and often need to be accompanied by adjuvants. The oil-based adjuvants included in the available fish vaccines can lead to severe adverse effects (covered in section 2.2.). The list of subunit- (both recombinant protein and VLP) and DNA vaccines under development for fish is long, but the success of these vaccines is dependent on adjuvant improvements (Tafalla et al., 2013).

2. Vaccine adjuvants

2.1. Development of adjuvants for use in humans

Non-living vaccine antigens (inactivated- and subunit vaccines) are often poorly immunogenic and will require development of adjuvants that can stimulate induction of protective humoral- and cell-mediated immunity (Coffman et al., 2010). Adjuvants (from latin *adjuvare* meaning “to help”) are a class of substances with a shared feature of increasing the immunogenicity and the efficacy of vaccines. The principle was first used by Gaston Ramon at Institute Pasteur in the 1920s and traditional adjuvants have been developed empirically, without a clear understanding of cellular and molecular mechanisms of action (Kenney and Cross, 2009). Vaccine adjuvants are a heterogeneous group of substances with a wide variety of mechanisms of action and recent research on vaccine development has focused on adjuvant improvement. Recent evidence suggests that adjuvants work through one or more of the following mechanisms: sustained release of

antigen, upregulation of cytokines and chemokines, increased antigen uptake and presentation, activation of inflammasomes, and activation, maturation and migration of antigen presenting cells (Cox and Coulter, 1997, Awate et al., 2013).

The dominating adjuvants in licensed vaccines are aluminium salts (aluminium hydroxide or aluminium phosphate). Although aluminium salts have been used in vaccines for over 80 years, their mechanism of action is not completely understood. Depot effect (sustained release) and inflammasome activation are among the proposed mechanisms (Kool et al., 2008, Marrack et al., 2009). However, it is well-known that aluminium adjuvants induce production of local cytokines and chemokines, increase cell recruitment and antigen presentation that induce a type 2 T helper cell (Th2) skewed response, and increase antibody production (Marrack et al., 2009, Awate et al., 2013). Other adjuvants are based on oils (often emulsions), liposomes, microparticles, surface-active agents, pathogen- and plant derivatives, vitamins, or cytokines. However, most of them yet to be included in licensed vaccines and remain in experimental use (Kenney and Cross, 2009).

The benefits of using an adjuvant are many, e.g. increased antibody titre and protective immunity, dose sparing, reduced number of immunisations (increased immunological memory), increased effect in populations with low response (e.g. elderly and children), enabling a more rapid immune response (post-exposure prophylaxis), antibody response broadening, and induction of a desired immune response against the specific pathogen (e.g. cell-mediated versus humoral response) (Coffman et al., 2010, Reed et al., 2013).

A shift from empiricism to rational design of adjuvants in human vaccinology research has led to several new, efficient adjuvants in vaccines that are already licensed or currently in clinical testing. A common feature of many new adjuvants under development is that they stimulate pattern recognition receptors (PRRs) expressed by innate immune cells. Various families of PRRs are identified, including Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), nucleotide oligomerization domain (NOD) like receptors (NLRs), and retinoic acid-inducible gene-1 (RIG-1) like receptors (RLRs) (Awate et al., 2013). These receptors sense conserved microbial features collectively called pathogen associated molecular patterns (PAMPs), and initiate innate immune responses as well as set the stage for an efficient adaptive immune response (Medzhitov, 2007). The best characterised family of PRR is the TLR family and their ligands' ability to induce inflammatory cytokines is explored for the immunostimulatory and adjuvant potential (Awate et al.,

2013, Steinhagen et al., 2011, Coffman et al., 2010). 3-O-desacyl-4'-monofosforyl lipid A (MPL) is a detoxified derivative of lipopolysaccharide (LPS) and ligand for TLR4. MPL is part of Glaxo Smith Kline's (GSK) Adjuvant System 04 (AS04) and is currently used in licensed vaccines against human papilloma virus and hepatitis B (Garçon et al., 2007). TLR ligands as potential vaccine adjuvants are covered in section 4.4.

2.2. Adjuvants for fish vaccines

The most common adjuvants in fish vaccines are based on mineral oil emulsions, which have been more or less unchanged since the development of these vaccines. These adjuvants increase immunogenicity of the antigen by making a depot at the injection site from which the antigen is slowly released (Tafalla et al., 2013). The emulsion usually consists of a water phase with the antigen dispersed in an oil phase (usually a mineral oil) with a surfactant (e.g. mannitol oleate) to stabilise the emulsion. The adjuvants registered under the trademark Montanide are based on mineral oil, non-mineral oil, or a mixture of both, and have been used in several commercialised fish vaccines. Freund's complete (FCA) and incomplete (FIA) adjuvants (known from human adjuvant research) consist of surfactant and paraffin oil with or without heat-killed mycobacteria. Both have been tested in experimental fish vaccines with variable results, but have not yet been used in commercial vaccines (Tafalla et al., 2013).

The side effects of oil-adjuvanted injection vaccines are undesirable for animal welfare reasons. Injectable vaccines formulated with oil-adjuvants can cause tissue lesions with granulomas at the injection site and abdominal cavity, adhesions between internal organs, autoimmunity reactions, reduced appetite and growth, and malfunction of reproductive organs (Koppang et al., 2004, Poppe and Breck, 1997, Håstein et al., 2005, Koppang et al., 2005, Haugarvoll et al., 2010, Midtlyng and Lillehaug, 1998). In addition to the type of adjuvants used, water temperature, fish size, hygiene during handling, time of the year, and anaesthesia can also influence the development and severity of side-effects (Berg et al., 2006, Håstein et al., 2005).

Although lagging behind the human adjuvant research, there are also promising adjuvant candidates for fish vaccines currently under development. For example, biocompatible and biodegradable nano- and microparticles offer a promising alternative to oil emulsions. The antigen can be covalently bound to or physically entrapped in these particles. Formulations based on polymers like poly-(lactide-co-glycolide) (PLGA) and chitosan are tested as

adjuvant systems in several fish species, both in injectable and oral vaccines (Plant and LaPatra, 2011, Tafalla et al., 2013). Trough cloning studies and fish genome projects, increasing knowledge about the fish innate immune system and pathogen recognition (e.g. TLRs) have made it possible to move to adjuvants with more specific mechanisms of action. Compounds like beta-glucans, cytokines and different PAMPs (e.g. TLR ligands), alone or in combination, are now studied as possible adjuvant candidates in fish vaccines (Tafalla et al., 2013, Dalmo and Bøgwald, 2008, Thim et al., 2014, Thim et al., 2012, Carrington and Secombes, 2006, Fredriksen and Grip, 2012, Rivas-Aravena et al., 2015). TLR ligands as potential vaccine adjuvants are covered in section 4.4.

3. Fish immune system

Fish is the largest class of vertebrates and can be divided into jawless fish and jawed fish, and the latter can be further divided in to cartilaginous fish (e.g. sharks) and bony fish (e.g. teleosts) (Hitzfeld, 2005). Bony fish (*Osteichthyes*) possess most of the components in the immune system associated with the mammals. Teleosts are a branch of bony fish to which most of the economically important species belong (e.g. salmonid, carp, and tilapia species). Zebrafish (*Danio rerio*), a species important in research, also belongs to the teleosts. In contrast to higher vertebrates, fish are free-living organisms from early embryonic stages of life in which they depend on their innate immune system for survival (Uribe et al., 2011).

3.1. Fish immune organs and cells

The immune organs of teleost fish differ from other vertebrates. Thymus, head kidney, and spleen are the main lymphoid organs in teleost, while bone marrow and lymph nodes are lacking. The head kidney (anterior part of the kidney) performs important immune function and is considered equivalent to the bone marrow in mammals. It also functions as a secondary lymphoid organ along with the spleen (Kaattari and Irwin, 1985, Kibenge et al., 2012). Gut-associated lymphoid tissue (GALT) is well developed in teleosts (Salinas, 2015), and a unique interbranchial lymphoid tissue (ILT) has been identified in salmonids (Koppang et al., 2010).

Teleost fish possess most of the immune cells known from the mammalian immune system; neutrophils, monocytes/macrophages, eosinophils, non-specific cytotoxic cells (similar to mammalian natural killer cells), and T and B lymphocytes (Rauta et al., 2012,

Secombes, 1996, Whyte, 2007). Epithelial cells may also be involved in innate defence in fish (Whyte, 2007). Dendritic-like cells have also been found in some fish species and may together with macrophages and B cells act as antigen-presenting cells (Bassity and Clark, 2012, Lugo-Villarino et al., 2010, Rauta et al., 2012). The main phagocytic cells in in fish are neutrophils and macrophages (Secombes and Fletcher, 1992). Respiratory burst and nitric oxide have been demonstrated in fish phagocytes (similar to homologous responses induced in mammalian phagocytes) and have been shown to be critical effector mechanisms in limiting the growth of fish pathogens (Neumann et al., 2001).

3.2. Components of fish innate immune system

The innate immunity is a fundamental defence mechanism in fish due to limitations of the adaptive immune system (Whyte, 2007). The physical barriers consist of skin, scales, and gills and represent the first line of defence against pathogens. The mucus covering the skin contains various components (e.g. lysozymes, IgM, antibacterial peptides, complement proteins, and lectins) which inhibit entry of pathogens (Uribe et al., 2011).

Fish secrete a wide range of antimicrobial peptides (i.e. in the saliva, mucus, and circulatory system) that play major roles in the innate immune system and protect against a variety of pathogens (Rajanbabu and Chen, 2011). The complement system of fish seems to have all of the three complement activation pathways known from the mammalian system. Compared to other vertebrates, fish possess a number of genes encoding several complement components that are structurally and functionally diverse, indicating the importance of this system in a rapid response against invading pathogens (Plouffe et al., 2005, Zhu et al., 2013)

A number of TLRs have been identified in teleosts, and a more detailed description of mammalian and fish TLRs is presented in section 4. Other PPRs found in fish are RLR and NLR families. The three mammalian members of the RLR have been identified in several fish species (e.g. rainbow trout, Atlantic salmon, and zebrafish); RIG-1 (Biacchesi et al., 2009, Nie et al., 2015), melanoma differentiation-associated gene 5 (MDA5) (also known as IFIH) (Sun et al., 2009, Chen et al., 2015, Chang et al., 2011a), and laboratory of genetics and physiology 2 (LGP2) (Chang et al., 2011a, Chen et al., 2015). In mammals, RLRs are responsible for detection of cytoplasmic viral RNA and they appear to be involved in antiviral immune responses in fish as well (Kawai and Akira, 2008, Poynter et al., 2015). NLRs are also cytoplasmic receptors and sense bacterial cell wall components in

mammals (Kanneganti et al., 2007). They are also present in several fish species (e.g. rainbow trout and zebrafish) and are most likely involved in antibacterial and antiviral defences (Laing et al., 2008, Chang et al., 2011b, Zhu et al., 2013). In zebrafish, the intracellular peptide glycan (PGN) recognition proteins (PGRPs) have been identified and may work together with TLR2 in recognition of PGN from bacteria (Chang and Nie, 2008).

Cytokines are a family of low molecular weight proteins that are secreted from immune cells (e.g. macrophages and lymphocytes) upon pathogen encounter to modulate inflammation and cope with pathogen infection. They can be divided into interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, and chemokines (Savan and Sakai, 2006). In general, fish possess a repertoire of cytokines similar to mammals (Reyes-Cerpa et al., 2012) and the most characterised ones in fish are the pro-inflammatory cytokines IL-1β and TNF-α (Plouffe et al., 2005).

IL-1β is a pro-inflammatory cytokine, one of the first cytokines to be upregulated during an infection, and has been found in many fish species (Secombes et al., 2011). The fish IL-1β share many of the characteristics with the mammalian counterpart, e.g. increases phagocytosis, chemotaxis, superoxide production, expression of important immune transcripts, leucocyte proliferation, and resistance to infection (Savan and Sakai, 2006, Plouffe et al., 2005, Hong et al., 2001, Peddie et al., 2001, Secombes et al., 2011). IL-1β activates target cells by binding to IL-1 receptors (IL-1R). Genes similar to the human IL-1R gene have been identified in rainbow trout and Atlantic salmon and were upregulated during LPS treatment (Sangrador-Vegas et al., 2000, Subramaniam et al., 2002). TNF-α has also been identified in numerous fish species and differ from the mammalian counterpart in the presence of multiple isoforms in some species (e.g. zebrafish and rainbow trout) (Reyes-Cerpa et al., 2012). Expression of TNF-α has been shown to increase during LPS stimulation in several fish species (Plouffe et al., 2005). While recombinant trout TNF-α has been shown to induce increased migration and phagocytic activity in trout head kidney leukocytes (Zou et al., 2003), in several other fish species the *in vitro* effects of TNF-α were surprisingly weak (Reyes-Cerpa et al., 2012). IL-6 is another important pro-inflammatory cytokine in the early mammalian immune response against pathogens, but little is known about its functions in fish. IL-6 can be upregulated by mimics of infection and seems to have similar effects as IL-1β in rainbow trout and

zebrafish (Costa et al., 2011, Varela et al., 2012). However, IL-6 downregulated IL-1β and TNF-α in trout head kidney macrophages, suggesting a potential role in limiting host damage during inflammation (Costa et al., 2011). IL-10 is known to be anti-inflammatory in humans and the gene is associated with suppression of Th1 response (Brocker et al., 2010). IL-10 has been identified in many fish species and while its role is not clear, it has been associated with mechanisms of immune evasion by IPNV in Atlantic salmon (Reyes-Cerpa et al., 2014). Chemokines are chemotactic cytokines that are involved in recruiting immune cells to the infection site. The most studied fish chemokine is IL-8, which has shown chemo-attractant properties in rainbow trout (Omaima Harun et al., 2008) and has been tested as an adjuvant in a VHSV DNA vaccine (Jimenez et al., 2006).

In mammals, interferons (IFNs) are the first line defence against viral infections. The large number of IFNs identified in vertebrates are grouped in type I (e.g. IFN-α and IFN-β), II (IFN-γ), and III (IFN-λ). Type I and III induce specific antiviral immune defences, while type II is involved in promoting cell-mediated immunity (Zou and Secombes, 2011). The nomenclature of fish IFNs has been controversial and several names and classifications exist. The type I IFN family of fish contains at least the four subtypes IFNa, IFNb, IFNc, and IFNd (Zou and Secombes, 2011). Atlantic salmon possess 11 virally induced IFN genes in a multiple gene cluster: two IFNa (IFNa1 and IFNa3), four IFNb (IFNb1–b4), and five IFNc (IFNc1–c5) (Sun et al., 2009). In addition, IFNa2 and IFNd has been found in Atlantic salmon, but outside the multiple gene cluster (Svingerud et al., 2012). Zebrafish also has an IFN gene cluster encoding IFNa1 (also called IFNϕ1/IFN1), IFNc1 and IFNc2 (also called IFNϕ2/IFN2 and IFNϕ3/IFN3, respectively), but no IFNb. In addition, a zebrafish IFNd1 (also called IFNϕ4) has been identified (Zou and Secombes, 2011, Hamming et al., 2011). Fish also possess homologues of mammalian type IIs (IFNγ) and these might be involved in both antiviral and –bacterial responses (Zou and Secombes, 2011). IFN expression is modulated by a family of transcription factors called interferon-regulatory factors (IRFs) which has been shown to exist in all vertebrates (Zhu et al., 2013). Type I IFNs work through IFN receptors to activate the Jak-Stat signalling pathway, of which all components have been identified in fish (Zhang and Gui, 2012, Levraud et al., 2007, Sun et al., 2014). This signalling pathway leads to expression of IFN-stimulated genes (ISGs) that exert numerous antiviral effector functions (Schoggins and Rice, 2011). Multiple ISGs have been identified in fish (e.g. ISG15 and Mx) that have shown to be virus-induced and exert antiviral activity in several fish species (Altmann et al., 2004,

Robertsen et al., 1997, Røkenes et al., 2007, Langevin et al., 2013, Jensen et al., 2002, Zhang and Gui, 2012). Furthermore, members of the ISG protein family IFIT (interferon-induced proteins with tetratricopeptide repeats) have shown antiviral function in zebrafish (Varela et al., 2014).

3.3. Brief overview of adaptive immunity in fish

The adaptive immunity can be divided into cell-mediated and humoral immunity. Fish seem to have lymphocyte subpopulations similar to the mammalian B and T cells and possess many important genes related to an adaptive immune response: MHC class I and II, T-cell receptor, CD4, CD8, and immunoglobulins. The presence of cytotoxic T cells (CD8+ cells) has been suggested (Nakanishi et al., 2011). Moreover, cytokines that in mammals are considered signature cytokines for Th1, Th2, and Th17 responses, have been identified in fish, and thus suggest the presence of Th1, Th2, and Th17 cells (Laing and Hansen, 2011). The main immunoglobulin in teleost is IgM, which has a heavy chain similar to the mammalian B cells. Fish IgM has a tetrameric structure (as opposed to the mammalian IgM pentameric structure) and is the primary antibody in fish serum (Solem and Stenvik, 2006). Additional immunoglobulin isotypes have also been identified in fish: IgD and IgT/IgZ (called IgT in rainbow trout (Hansen et al., 2005) and IgZ in zebrafish (Danilova et al., 2005)). IgT may be important in the GALT of rainbow trout, thus perhaps representing a functional analogue to IgA (Zhang et al., 2010). The existence of B cells with phagocytic and bactericidal activity has been suggested (Sunyer, 2012). Isotype switch has not been described in fish and the antibody response is generally known for being slow, having low affinity, and being temperature dependent (Sunyer, 2012). However, there are several examples showing that fish are able to induce specific and strong antibody responses after pathogen challenge or vaccination, and that antibody levels can be used as a correlate of protection (Munang'andu et al., 2013, Solem and Stenvik, 2006, Fjalestad et al., 1996, Steine et al., 2001, Thim et al., 2012).

4. Toll-like receptors (TLRs)

4.1. Mammalian TLRs and their ligands

In human vaccinology, the TLRs and their ligands have been extensively studied. TLRs are, as previously mentioned, important receptors that sense invading pathogens (O'Neill et al., 2013). TLRs appeared in the early stages of evolution and have been conserved in both

invertebrate and vertebrate lineages (Medzhitov and Janeway, 2000). The first evidence of the Toll NF-κB-like signalling pathway was discovered when the Toll protein was found to be required for fungal resistance in fruit flies (*Drosophila melanogaster*) (Lemaitre et al., 1996) and a short time later the human homolog of Toll was found (Medzhitov et al., 1997). Since then, TLRs have been described in a wide variety of vertebrate species. Six major TLR families (TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11) have been identified, and TLRs within a family recognise a general class of PAMPs associated with the family (Roach et al., 2005). TLRs are transmembrane proteins containing an extracellular recognition domain composed of multiple leucine-rich region (LRR) motifs, a transmembrane region, and an intracellular Toll/interleukin-1 receptor (TIR) signalling domain (named TIR because the similarity of the IL-1R signalling domains) (Botos et al.). Upon binding a ligand, the TLRs are relocated into the lipid raft fraction of the cell membrane (Sadikot, 2012) before two TLRs dimerise (either heterodimerisation or homodimerisation) for firm ligand binding (Jin and Lee, 2008). The close proximity of the TIR domains of paired TLRs allows recruiting of TIR domain-containing adaptor proteins. These adaptors are Myeloid differentiation primary response gene (88) (MyD88), Mydd88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM, also known as TICAM2), or sterile α-and armadillo-motif-containing protein (SARM) (O'Neill and Bowie, 2007). The engagement of the adaptor molecules stimulates downstream intracellular signalling pathways. These pathways involve interactions between IL-1R-associated kinases (IRAKs) and TNF-receptor-associated factors (TRAFs) and will eventually lead to activation of transcription factors (nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs), cyclic AMP-responsive element-binding protein (CREB), or activator protein 1 (AP1)) that control hundreds of different immune-relevant genes. The intracellular signalling cascade is complex and several other factors are involved; however, not all of them can be described in detail here. The adaptor molecules involved, the signalling pathway induced, and the cytokine expression profile following stimulation of a TLR, depend on the type of pathogen and the TLRs recognising the pathogen, as shown in Figure 2 (O'Neill et al., 2013). The transcription factors activated lead to upregulation of pro-inflammatory cytokines (e.g. IL-β, TNF-α, and IL-6) involved in inflammation and/or Type I IFNs (IFN-α, IFN-β) involved in antiviral immune response (see Figure 2).

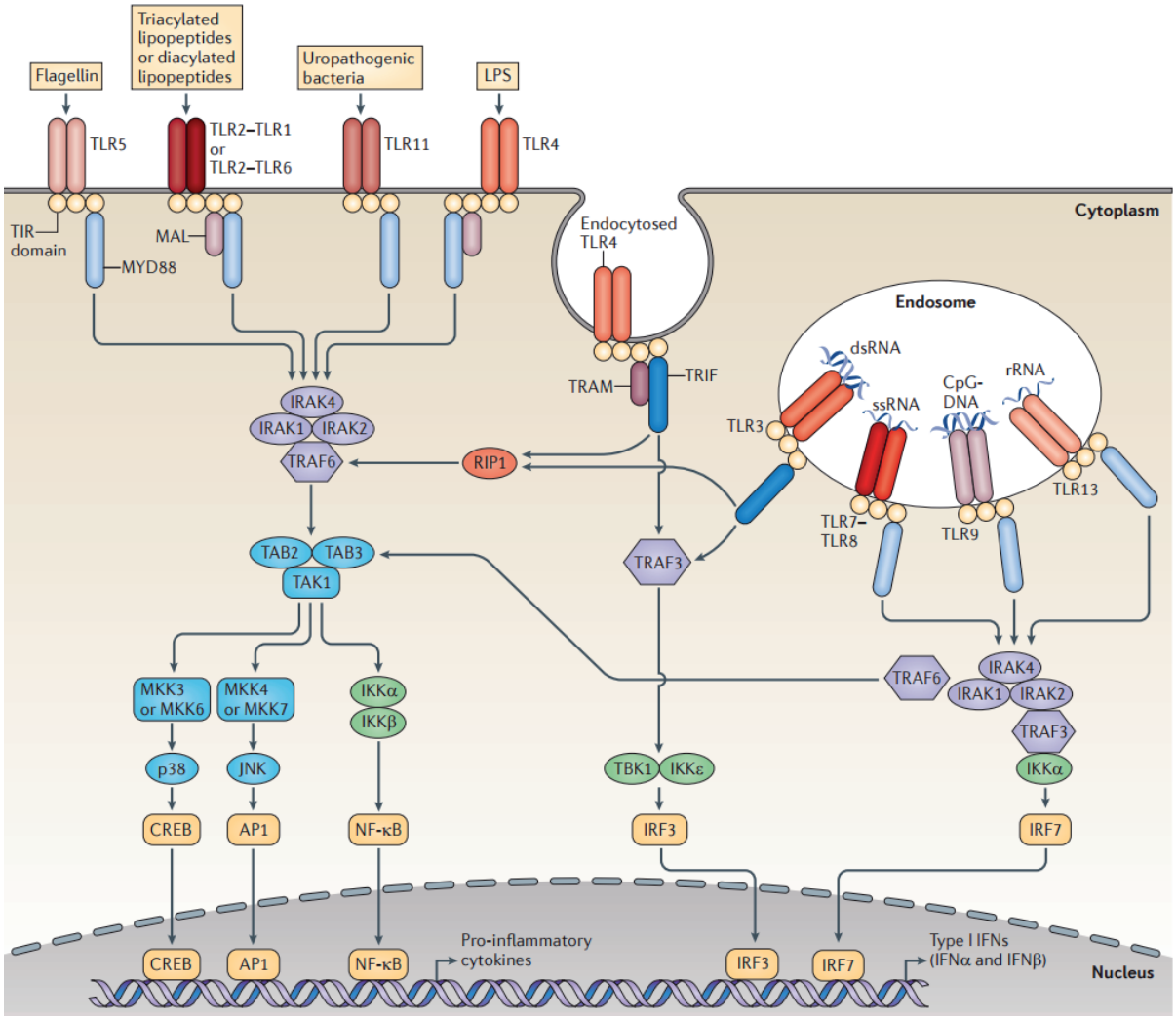

Figure 2: *Overview of mammalian TLR signalling pathways. Figure from (O'Neill et al., 2013).*

The diversity of the LRR folding defines the TLR binding specificities and will orchestrate the appropriate innate and adaptive immune responses against the specific pathogen. TLR1, TLR2, TLR4, TLR5, and TLR6 are localised on the cell surface (TLR4 can also be found in endosomes) and recognise cell wall PAMPs, while TLR3, TLR7, TLR8, and TLR9 are localised in membranes of intracellular compartments (like endosomes) and recognise nucleic acids. Bacterial PAMPs are mainly sensed by TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9, while viral PAMPs are sensed by TLR3, TLR7, TLR8, and TLR9 (Jin and Lee, 2008, Werling et al., 2009). An overview of mammalian TLRs and their ligands is presented in Table 2.

Table 2: Mammalian TLRs and their ligands

TLR	Species	Localisation	Ligand examples
TLR1/TLR2	Human Mouse	Plasma membrane	Bacterial triacylated lipopeptides Mycobacterial products Porins Synthetic triacylated lipopeptides (e.g. Pam3CSK4)
TLR2/?	Human Mouse	Plasma membrane	Bacterial lipoproteins *Staphylococcus* peptidoglycan Viral proteins (from certain viruses)
TLR3	Human Mouse	Endosomal membrane	Viral double stranded RNA PolyI:C
TLR4 (+ MD-2 and CD14)	Human Mouse	Plasma and endosomal membrane	Lipopolysaccharide (LPS) Lipid A derivatives (e.g. monophosphoryl A (MPL)) Viral proteins (from certain viruses)
TLR5	Human Mouse	Plasma membrane	Bacterial flagellin Recombinant flagellin
TLR2/TLR6	Human Mouse	Plasma membrane	Bacterial diacylated lipopeptides Lipoteichoic acid Synthetic diacylated lipopeptides (e.g. FSL-1, Pam2CSK4)) Zymosan
TLR7	Human Mouse	Endosomal membrane	Viral and bacterial single stranded RNA Thiazoquinolines Imidazoquinolines (e.g. imiquimod)
TLR8	Human Mouse	Endosomal membrane	Viral and bacterial single stranded RNA Thiazoquinolines Imidazoquinolines (e.g. resiquimod)
TLR9	Human Mouse	Endosomal membrane	Viral and bacterial CpG DNA DNA:RNA hybrids Class A, B and C CpG oligodeoxynucleotides (e.g.ODN2006)
TLR10	Human	Plasma membrane	Unknown
TLR11	Mouse	Endosomal membrane	Profilin, flagellin
TLR12	Mouse	Endosomal membrane	Profilin
TLR13	Mouse	Endosomal membrane	Bacterial ribosomal RNA, vesicular stomatitis virus

References (De Nardo, 2015, Oliviera-Nascimento et al., 2012, Bowie and Haga, 2005, Shi et al., 2011)

Mammalian TLR2 requires heterodimerisation with TLR1 or TLR6 and is involved in recognition of bacterial and fungal cell wall components. Homodimerisation of TLR2 has been suggested, but not confirmed (Oliviera-Nascimento et al., 2012). Several synthetic compounds that mimic bacterial lipoproteins (e.g. Pam3CSK3 and FSL-1) are well established agonists for mammalian TLR1/2 and TLR2/6 (Okusawa et al., 2004, Aliprantis et al., 1999, Ozinsky et al., 2000). TLR4 recognises Gram-negative bacteria via the lipid A part of LPS (Poltorak et al., 1998). The recognition of LPS also requires the co-receptors MD-2 and CD14, and the LPS-binding protein (LBP) facilitates the transfer of LPS to CD14 (Park and Lee, 2013). TLR4 is unique in being able to signal trough both Myd88/MAL and TRIF/TRAM. It uses the adaptor TRAM to recruit TRIF and induce IRF3 activation and IFN-β expression, but can also use MAL to recruit Myd88, which

leads to activation of NF-κB and AP-1 that will induce pro-inflammatory cytokines (Thompson and Locarnini, 2007). In addition, TLR2 and TLR4 have been shown to interact with viral components (e.g. viral glycoproteins) and may participate in in viral detection (Bowie and Haga, 2005).

TLR3 is responsible for sensing viral double-stranded RNA (dsRNA) (Alexopoulou et al., 2001) and is the only TLR that works via the Myd88-independent pathway. The adaptor TRIF is instead essential for TLR3-mediated signalling. TRIF interacts with TRAF3 to activate a kinase cascade that leads to the activation of IRF3, which induces expression of IFN-β. TRIF can also interact with TRAF6 to induce a late phase NF-κB and pro-inflammatory cytokine response (Thompson and Locarnini, 2007, Jensen and Thomsen, 2012). Polyinosine–polycytidylic acid (poly I:C) is a synthetic analogue of dsRNA that binds TLR3 and is extensively used to mimic a viral infection (Fortier et al., 2004). The natural ligand for human TLR7 and TLR8 is single-stranded RNA (ssRNA) (Heil et al., 2004, Lund et al., 2004). In addition, a group of synthetic antiviral-RNA-like compounds (e.g. imiquimod) work by binding TLR7 and TLR8 (Hemmi et al., 2002, Lee et al., 2003a). Human TLR9 recognises bacterial and viral DNA, which typically contain un-methylated CpG oligodeoxynucleotides (ODNs) in higher frequencies than human DNA (Hemmi et al., 2000). TLR7, TLR8, and TLR9 signal trough Myd88 and TRAF6 that lead to activation of both NF-κB to upregulate pro-inflammatory cytokines and IRF7 to upregulate IFN-α and IFN-β (Thompson and Locarnini, 2007, Jensen and Thomsen, 2012).

4.2. TLRs in fish

The advances in fish genomic research during the last decade have led to the discovery of TLR genes in many species of bony fish. Ever since the first teleost TLR was discovered in goldfish macrophages (Stafford et al., 2003), about 20 TLR types (TLR1, 2, 3, 4, 5M, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25, and 26) have been identified in more than a dozen of teleost species (Rauta et al., 2014). The fish TLRs and the factors involved in their signalling cascade have high structural similarity to the mammalian TLR system. Figure 3 shows a phylogenetic tree comparing full-length TLR amino acids sequences from Atlantic salmon, rainbow trout, and zebrafish with the human and mice TLRs. The tree shows the homology of the different TLRs between the different species, and that the TLRs make up six TLR families. Most vertebrate genomes are actually found to have at least one gene representing each of the six major TLR families (Roach et al., 2005).

Although fish TLRs have high structural similarity with mammalian TLRs and possess many homologues of the mammalian TLRs, they also have distinct features and differences. Some mammalian TLRs have yet to be found in fish and several non-mammalian and fish-specific TLRs have been identified (Palti, 2011). In many fish species (e.g. Atlantic salmon and rainbow trout), a soluble form of TLR5 (TLR5S) has been identified in addition to the membrane-bound form (TLR5M). Two putative soluble forms of TLR20 have also been found in Atlantic salmon (Lee et al., 2014). Other non-mammalian TLR genes found in several fish species are TLR14 and TLR18 that branches with the TLR1 family, and TLR19-26 that belongs to the TLR11 family alongside mouse TLR11-13 (see Figure 3). Some of these TLRs are unique to fish (e.g. TLR22), while others have only been found in aquatic animals (e.g. TLR14) (Rauta et al., 2014). Orthologues of TLR6 and TLR10 seem to be absent from fish genomes, but TLR14 and TLR18 have been proposed as possible substitutes (Zhang et al., 2014). Zebrafish is one of the few fish species in which TLR4 has been identified (Meijer et al., 2004, Jault et al., 2004). Fish TLR21 has shown similarity to chicken TLR21, which is considered a functional homologue to mammalian TLR9 (Brownlie et al., 2009). Genome- and gene duplication events have been contributors to the diversity of the TLRs in fish and a number of duplicate multi-copy TLRs have been identified (e.g. Atlantic salmon TLR8a1, TLR8a2, TLR8b1, and TLR8b2) (Palti, 2011).

Unc93B1 is a chaperone that appears to be important for the trafficking of endosomal TLRs (TLR3, TLR7, TLR8, TLR9, and TLR11-13) within the mammalian cell (Gay et al., 2014). The gene has been identified in Atlantic salmon and zebrafish, and is thought to have a role in fish TLR signalling (Yeh et al., 2013, Lee et al., 2015). Most of the downstream molecules involved in TLR signalling have also been identified in fish and the pathways seem to be conserved. However, information on the functional importance of many of these genes is lacking (Rebl et al., 2010, Kanwal et al., 2014). Myd88 has been identified in Atlantic salmon, rainbow trout, and zebrafish, and seems to function similarly to the mammalian counterpart (Skjæveland et al., 2009, van der Sar et al., 2006, Iliev et al., 2011, Rebl et al., 2009). Furthermore, MAL, TRIF, IRAK4, TRAF6, NF-κB, IRF3, and IRF7 have also been identified in fish (Phelan et al., 2005, Brietzke et al., 2014, Iliev et al., 2011, Meijer et al., 2004, Kanwal et al., 2014, Purcell et al., 2006); but TRAM has not been identified in any fish to date (Zhang et al., 2014).

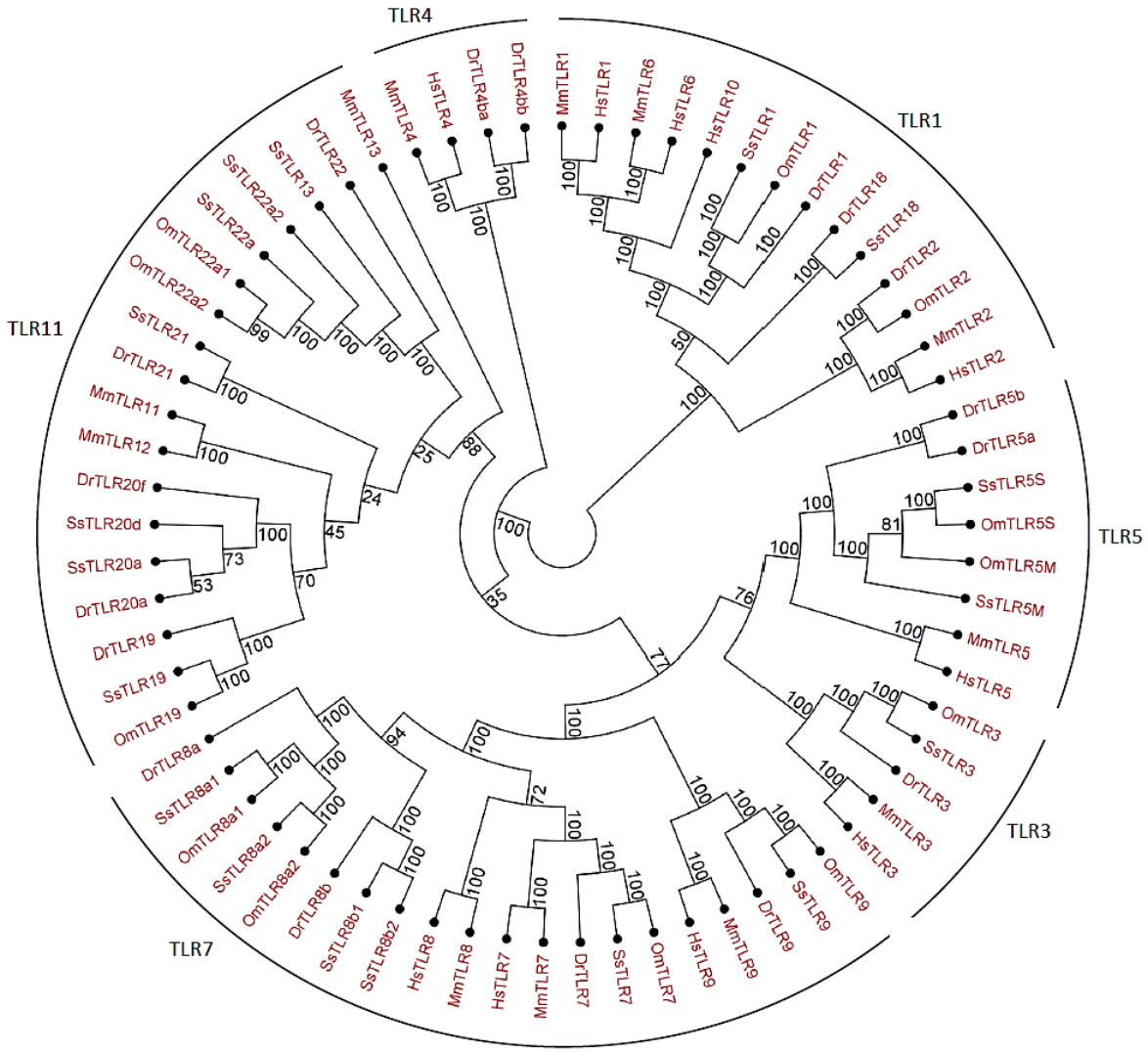

Figure 3: *Circular phylogenetic tree of full-length TLR amino acid sequences of Atlantic salmon (Salmo salar, Ss), Rainbow trout (Oncorhynchus mykiss, Om), Zebrafish (Danio rerio, Dr), human (Homo sapiens, Hs) and mouse (Mus musculus, Mm). Bootstrap values based on 100 replicates are indicated on each branch. Accession numbers of sequences used to build the three are as follows: Danio rerio (TLR1: AAI63271, TLR2: NP_997977, TLR3: NP_001013287, TLR4ba: NP_001124523, TLR4bb: NP_997978, TLR5a: XP_001919052, TLR5b: NP_001124067, TLR7: XP_003199309, TLR8a: XP_001920594, TLR8b: XP_003199440, TLR9: NP_001124066, TLR18: AAI63840, TLR19: AAQ91317, TLR20a: AAI63786, TLR20f: AAQ91319, TLR21: NP_001186264, TLR22: NP_001122147); Homo sapiens (TLR1: NP_003254, TLR2: NP_003255, TLR3: NP_003256, TLR4: NP_612564, TLR5: NP_003259, TLR6: NP_006059, TLR7: NP_057646, TLR8: NP_619542, TLR9: NP_059138, TLR10: NP_001017388); Mus musculus (TLR1: NP_109607, TLR2: NP_036035, TLR3: NP_569054, TLR4: NP_067272, TLR5: NP_058624, TLR6: NP_035734, TLR7: NP_573474, TLR8: NP_573475, TLR9: NP_112455, TLR11: NP_991388, TLR12: NP_991392, TLR13: NP_991389); Oncorhynchus mykiss (TLR1: NP_001159573, TLR2: CCK73195, TLR3: NP_001118050, TLR5S: NP_001118216, TLR5S: P_001117680, TLR7: ACV41797, TLR8a1: ACV41799, TLR8a2: ACV41798, TLR9: ACC93939, TLR19: CCW43211, TLR22a1: NP_001117884, TLR22a2: CAI48084); Salmo salar (TLR1: AEE38252, TLR3: AKE14222, TLR5M: AEE38253, TLR5S: AEE38254, TLR7: CCX35457, TLR8a1: NP_001155165, TLR8a2: CCX35458, TLR8b1: CCX35459, TLR8b2: CCX35460, TLR9: ABV59002, TLR13: NP_001133860, TLR18: CDK60413, TLR19: CDH93609, TLR20a: CDH93610, TLR20d: CDH93613, TLR21: CDH93614, TLR22a: CAJ80696, TLR22a2: CAR62394).Figure made by M.Arnemo with CLC Main Workbench7.*

4.3. Ligand specificity of fish TLRs

Many functional studies have failed to detect ligand specificities for fish TLRs. Direct ligand specificity has only been reported for a few TLRs, using *in vitro* reporter assays to detect ligand recognition (Ribeiro et al., 2010, Matsuo et al., 2008, Tsujita et al., 2004, Yeh et al., 2013). However, numerous published stimulation and gene expression analyses discuss possible ligand specificities and roles of fish TLRs during infection. The studies concerning Atlantic salmon, rainbow trout, and zebrafish will be reviewed here.

Since TLR6 has yet to be found in most fish genomes, TLR1 is the most likely partner for heterodimerisation with TLR2 in fish (Pietretti and Wiegertjes, 2013). Few reports are available on salmonid TLR1 and TLR2, but bacterial infection has shown to upregulate TLR1 *in vitro* (Salazar et al., 2015), while Pam2CSK4 and Pam3CSK4 (classical ligands for human TLR2/6 and TLR1/2, respectively) seemed to have a lower potential for inducing TLR and cytokine expression (Palti et al., 2010b, Purcell et al., 2006). Zebrafish TLR18 branches with the TLR1 family and was upregulated together with TLR1 and TLR2 during *M.marinum* infection (Meijer et al., 2004). Atlantic salmon, rainbow trout, and zebrafish TLR3 have been cloned and characterised by expression analysis, which demonstrated upregulation of TLR3 and type I IFNs following infection with aquatic viruses or poly I:C stimulation (Phelan et al., 2005, Vidal et al., 2015, Svingerud et al., 2012, Purcell et al., 2006, Rodriguez et al., 2005, Jensen et al., 2002, Dios et al., 2010). This indicates conservation of TLR3-signalling pathways as well as involvement in antiviral immunity and binding of dsRNA.

The high tolerance of LPS in fish was for a long time explained by the lack of TLR4 in most fish genomes (e.g. Atlantic salmon and rainbow trout). This explanation was challenged when two TLR4 genes (TLR4ba and TLR4bb) were identified in zebrafish (Meijer et al., 2004, Jault et al., 2004). However, the apparent absence of CD14, MD-2, and LBP from all fish genomes may play a role (Pietretti and Wiegertjes, 2013). Although zebrafish tolerate high doses of LPS, LPS has been shown to exert multiple biological effects (Novoa et al., 2009, Swain et al., 2008). It seems that zebrafish are responsive to LPS through a TLR4-independent pathway, thus suggesting an alternative LPS induction pathway in fish (Sullivan et al., 2009, Sepulcre et al., 2009). Aedo et al. (2015) proposed that TLR5M and TLR9 may play a role in detecting LPS in rainbow trout (Aedo et al., 2015). Conservation of flagellin binding by TLR5 has been suggested in rainbow trout,

Atlantic salmon, and zebrafish. Both forms of rainbow trout TLR5 (soluble and membrane-bound), in a chimeric form with TIR domain from human TLR5, stimulated NF-κB activation *in vitro* after exposure to flagellin (Tsujita et al., 2004). Flagellin has also been shown to induce upregulation of pro-inflammatory cytokines and both TLR5 forms *in vitro* and *in vivo* in salmonids (Hynes et al., 2011, Purcell et al., 2006, González-Stegmaier et al., 2015). In zebrafish, the two TLR5 isoforms (TLR5a and TLR5b) were upregulated in several studies during bacterial infections and flagellin stimulation (Meijer et al., 2004, Stockhammer et al., 2009, van der Sar et al., 2009, Yang et al., 2013). Additionally, morpholino-induced TLR5 zebrafish knockdowns led to a reduction of flagellin-induced inflammation (Yang et al., 2015).

Upregulation of type I interferons and pro-inflammatory cytokines have been demonstrated by stimulation with TLR7 and TLR8 ligands in rainbow trout (*in vitro*) and Atlantic salmon (*in vitro* and *in vivo)* (Purcell et al., 2006, Kileng et al., 2008, Palti et al., 2010a, Svingerud et al., 2012). Type I interferons upregulated TLR7 and TLR8 variants in Atlantic salmon (Lee et al., 2013), while *in vivo* viral infections both induced or not affected TLR8 expression (Skjæveland et al., 2009, Skjesol et al., 2011). Stimulation by CpG ODNs has been studied a lot in salmonids, both *in vivo* and *in vitro*. CpG ODNs have been shown to both upregulate and not affect TLR9 expression (Ortega-Villaizan et al., 2009, Skjæveland et al., 2008, Strandskog et al., 2008), while pro-inflammatory cytokines, type I IFN, and ISGs usually are upregulated by these PAMPs (Strandskog et al., 2008, Jørgensen et al., 2001, Jørgensen et al., 2003, Carrington and Secombes, 2006). Bacterial infections have been shown to induce zebrafish TLR9 expression (Meijer et al., 2004, Uma et al., 2012) and TLR9 has been demonstrated to recognise CpG ODNs in a NF-κB-reporter assay (Yeh et al., 2013).

The non-mammalian TLRs have also been investigated in several infection and stimulation studies. It has been proposed that TLR3 may not be the only TLR that senses viral dsRNA in fish, as fugu TLR22 was demonstrated to recognise long-sized dsRNA when tested for IFN-β activation in an *in vitro* reporter assay (Matsuo et al., 2008). Binding of dsRNA has also been suggested for zebrafish TLR22 (Sahoo et al., 2014). Bacterial infections have been shown to upregulate TLR22 in rainbow trout and zebrafish (Meijer et al., 2004, Rebl et al., 2007). In addition, poly I:C, PGN, and LPS have been shown to upregulate zebrafish TLR22 (Sundaram et al., 2012). In the same study, these TLR ligands also upregulated

zebrafish TLR21, which later was shown to recognise CpG ODNs in a NF-κB-reporter assay (Yeh et al., 2013). TLR20 has been suggested a role in immune response to protozoan parasites in fish, similar to the murine TLR11 and TLR12 (Pietretti et al., 2014, Zhang et al., 2014). However, Atlantic salmon TLR18-21 were mostly unaffected or downregulated during ISAV infection (Lee et al., 2014), thus the functional role of many of these TLRs is not yet clear.

4.4. TLR ligands as vaccine adjuvants

Due to the TLR ligands' ability to induce inflammatory cytokines and important immune mediators, these ligands are extensively explored for their adjuvant properties in human vaccines, and the list of TLR ligands in clinical trials is long (Toussi and Massari, 2014, Steinhagen et al., 2011, Tomai and Vasilakos, 2012). With more knowledge about the existence of fish TLRs, research on these ligands in fish vaccinology is also increasing (Tafalla et al., 2013).

The TLR4 ligand LPS and its derivatives are the most widely tested TLR ligands in human vaccines. Due to LPS toxicity, its use in human vaccines was limited until detoxified variants were developed. The TLR4 agonist MPL, a low-toxicity derivative of LPS, is a component that together with aluminium hydroxide is included in the GSK adjuvant AS04. Aluminium salt adjuvants are known for inducing Th2-dominating immune responses, but when combined with MPL, Th1 responses are induced. This makes AS04 especially useful for vaccines against intracellular pathogens (Duthie et al., 2011, Didierlaurent et al., 2009). Poly I:C and CpG ODNs, ligands for TLR3 and TLR9, respectively, have proven effective as vaccine adjuvants in clinical trials. Because of poly I:C's potent antiviral activity and mimicking of viral infection, there are numerous studies on its immunostimulatory potential in therapeutics or vaccines available (Hafner et al., 2013). Similar to other dsRNA complexes, poly I:C leads to induction of IL-12 and type I IFN, and it can promote Th1 based immunity, development of CD8+ T cells, and enhance antibody production against several viral antigens (e.g. influenza, HIV, and hepatitis B) (Toussi and Massari, 2014, Tomai and Vasilakos, 2012). Lipopeptides (e.g. Pam3CKS) and flagellin have also reached clinical trials, but most of the knowledge about these ligands results from pre-clinical models. Their effect is characterised predominantly as Th2-biased and are typically tested out in bacterial vaccines. Agonists for TLR7 and TLR8, are less developed as

vaccine adjuvants, but are in use in topical formulations in antiviral and cancer immunotherapy (Toussi and Massari, 2014).

As reviewed in section 4.3., several TLR ligands seem to have immunostimulatory properties in fish. The non-specific antiviral effect has been tested for several TLR ligands in fish. For example, poly I:C and CpG have been shown to induce an antiviral state strong enough to confer resistance to viral challenge in several fish species (Ruyra et al., 2014, Jørgensen et al., 2003, Jensen et al., 2002, Kim et al., 2009, Nishizawa et al., 2009, Oh et al., 2012, Takami et al., 2010). Although several studies focus on the non-specific immunostimulatory properties of these ligands, there are few reports available on the adjuvant effect together with an antigen (Tafalla et al., 2013, Carrington and Secombes, 2006). However, the combination of poly I:C and CpG as adjuvant in an inactivated SAV vaccine have shown promising results as adjuvant strategy (Thim et al., 2012, Thim et al., 2014, Strandskog et al., 2008).

5. Dietary n-3 fatty acids and immune responses

Fish oil is the major source of the very long-chain n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Due to increasing demand for EPA and DHA as ingredients in human health products and fish feed, there is now a shortage of these fatty acids on the international markets. This has led to increased substitution of fish oil by plant oils in Atlantic salmon feed (Figure 4), thereby reducing the EPA and DHA content of salmon.

EPA and DHA are well known to modulate inflammation and immune responses in humans and experimental animals, and their main effects are generally recognised to be anti-inflammatory (Hummell, 1993, Calder, 2013). The composition of fatty acids in membrane phospholipids of mammalian immune cells strongly correlate with the profile of dietary lipids (Calder, 2013). EPA and DHA have been suggested to modulate TLR function trough interfering with lipid rafts and signalling platforms in the cell membrane of mammalian immune cells (Calder, 2013, Lee et al., 2003b, Norris and Dennis, 2012).

Also in fish, varying the dietary levels of EPA and DHA has been shown to alter the fatty acid composition in organs rich in immune cells like the head kidney (Bell et al., 1993, Hvattum et al., 2000, Ganga et al., 2005, Bell et al., 1998). Since membrane phospholipids are a part of the microenvironment for leucocyte transmembrane signalling receptors (e.g.

TLRs), the effects of dietary EPA and DHA may have consequences for leucocyte function and fish health (e.g. clearance of pathogens and vaccine effects).

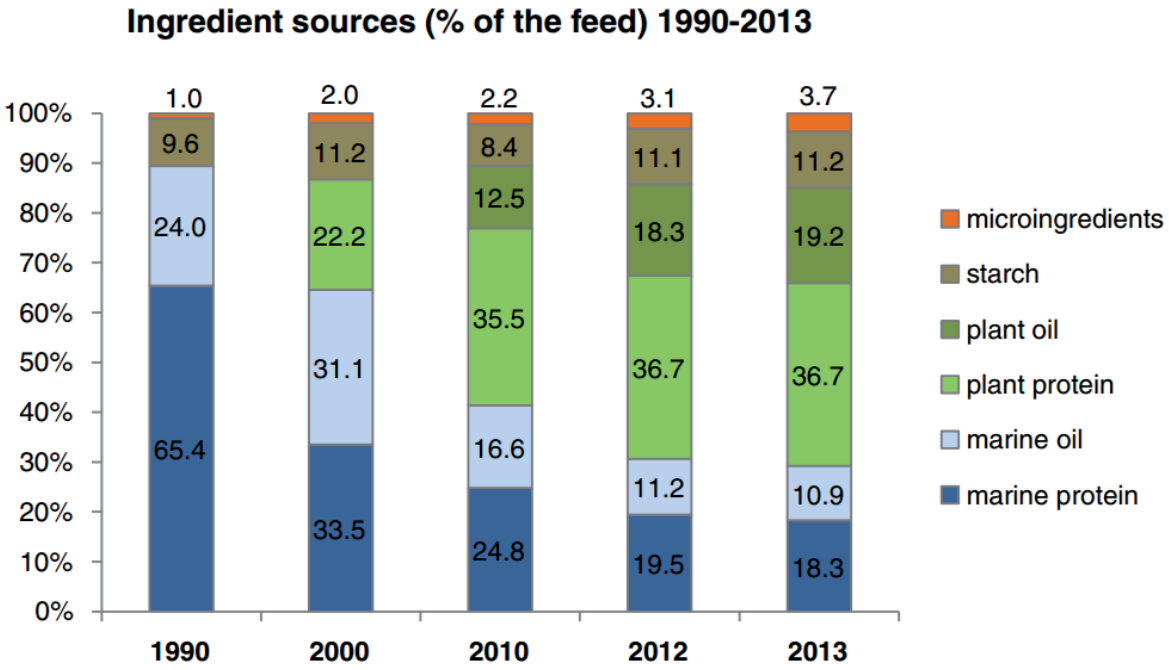

Figure 4: *Nutrient sources in Norwegian salmon farming from 1990 to 2013. Each ingredient type is shown as its percentage of the total diet. In 2013, only 30% of the ingredients were of marine origin compared to 90% in 1990. Figure from (Ytrestøyl et al., 2015).*

Investigations of the effects of low n-3 fatty acid in diet or cell media have demonstrated varying results on immune cell function and diseases resistance in fish (Montero and Izquierdo, 2010). The replacement of fish oil by vegetable oil has demonstrated to impair macrophage function (e.g. phagocytosis and respiratory burst) in several studies (Kiron et al., 1995, Montero et al., 2003, Montero et al., 2008, Sheldon and Blazer, 1991). On the contrary, other papers reported no effects on immune cell function of this replacement (Seierstad et al., 2009, Gjøen et al., 2004). Regarding disease resistance *in vivo*, both decreased, increased and no effects on mortality have been demonstrated in fish (Thompson et al., 1996, Waagbø, 1994, Gjøen et al., 2004, Lødemel et al., 2001, Bransden et al., 2003). Dietary lipids can obviously affect immune functions in fish, however, it the role of these lipids on fish health is not clear. In addition, little is known about the molecular mechanisms.

Aims of the thesis

In human vaccinology, the TLRs are studied in great detail and their ligands are explored for their adjuvant and immunostimulatory properties. This research has led to several new, efficient adjuvants. There is a need for new adjuvants in fish vaccines. To benefit what is known from human adjuvant research, a greater molecular understanding and functional description of the similarities and differences between mammalian and fish innate immunity is needed. Therefore, increased knowledge of fish TLR function is required before their ligands can be used in fish vaccines as adjuvants.

Main objective:

Contribute to a more detailed understanding of fish TLRs.

Sub-objectives:

1. Provide an overview over TLR expression and function in Atlantic salmon by investigating tissue distribution of TLRs, the impact of viral infection on TLR expression, and the cytokine induction potential of TLR ligands in primary leucocytes.
2. Functionally validate ligand-specific activation of fish TLRs by using an *in vitro* reporter assay.
3. Test the *in vivo* immunostimulatory potential of a TLR ligand in zebrafish.
4. Test the adjuvant effects of a TLR ligand in virus vaccines *in vivo* in zebrafish.
5. Investigate if various dietary levels of EPA and DHA can affect TLR signalling in Atlantic salmon.

Summary of papers

Paper I: Effects of TLR agonists and viral infection on cytokine and TLR expression in Atlantic salmon (*Salmo salar*).

Arnemo M, Kavaliauskis A, Gjøen T.

Developmental and Comparative Immunology (2014)

To provide an overview of TLR expression and function in Atlantic salmon, we used primers of all known Atlantic salmon TLRs at the time to investigate tissue distribution of TLRs, the immunostimulatory potential of classical TLR ligands in primary leucocytes (*in vitro*), and the impact of viral infection on TLR expression (*in vivo*). Head kidney and spleen were the main organs expressing TLRs in Atlantic salmon. Adherent head kidney leucocytes were isolated and stimulated with a panel of known ligands for human TLR1-9. The cells were able to respond to many of the ligands in terms of upregulating pro-inflammatory cytokines, IFNs, and Mx. Poly I:C and imiquimod induced expression of IFNs and Mx, which are important in antiviral immunity. TLR3, TLR7, and TLR8a1 were upregulated in head kidney of ISAV infected fish.

Paper II: Structurally diverse genes encode TLR2 in rainbow trout: The conserved receptor cannot be stimulated by classical ligands to activate NF-kappaB in vitro.

Brietzke A, Arnemo M, Gjøen T, Rebl H, Korytář T, Goldammer T, Rebl A, Seyfert HM.

Developmental and Comparative Immunology (2016)

We wanted to establish a functional assay to screen for fish TLR ligand specificity. The mammalian TLR2 is important in recognition of bacterial cell wall components, but has also been demonstrated to sense certain viral proteins. Alexander Rebl and his group obtained the full-length cDNA sequence encoding TLR2 from rainbow trout (*Oncorhynchus mykiss*, Om). The primary structure of the encoded receptor complied with the domain structure and ligand-binding sites known from mammals and other fish species. OmTLR2a was expressed both in HEK-293 and salmon CHSE-214 cells together with an NF-kB luciferase reporter plasmid. Regarding the CHSE-214 cell assay, we first validated that poly I:C and flagellin, but not FSL-1 or Pam3CSK4 stimulation activated NF-kB in these cells. However, no significant ligand-dependent activation of NF-kB could be detected in TLR2a-transfected CHSE-214. Neither synthetic lipopeptides known to

stimulate mammalian TLR2, nor exposure to different bacteria induced OmTLR2-mediated activation.

Paper III: Use of poly I:C stabilised with chitosan as a vaccine-adjuvant against Viral Haemorrhagic Septicaemia Virus infection in zebrafish.
Kavaliauskis A, Arnemo M, Kim SH, Ulanova L, Speth M, Novoa B, Dios S, Evensen Ø, Griffiths GW, Gjøen T.
Zebrafish (2015).

There is a need for new adjuvants and more efficient viral vaccines in fish farming. By using zebrafish as a model system, we could test the *in vivo* immunostimulatory and antiviral resistance properties of poly I:C. In addition, the adjuvant effect of poly I:C stabilised with chitosan could be tested in an inactivated VHSV vaccine. Poly I:C proved to be a strong inducer of the antiviral state, measured by transcriptional activation of the genes of viral sensors and effectors (TLRs, IFNs, and ISGs), and delayed VHSV-induced mortality when injected 24 hours pre-challenge. Poly I:C was stabilised with chitosan and co-injected with inactivated VHSV (CSpIC+iV vaccine) in adult zebrafish. The protective effect against VHSV was compared to a live attenuated VHSV vaccine (aV). Both CSpIC+iV and aV formulations provided protection against VHSV-induced mortality. However, when plasma from survivors was tested for neutralizing antibodies in an *in vitro* protection assay, we could not demonstrate any protective effect. Plasma from aV vaccinated fish enhanced cytopathic effects, indicating that antibody-dependent entry may play a role in this system.

Paper IV: Chitosan-poly I:C nanoparticles: a novel adjuvant in aquaculture vaccines. A study of particle bio- distribution and immune response in zebrafish (*Danio rerio*).
Kavaliauskis A, Arnemo M, Speth MT, Lagos Rojas LX, Rishovd AL, Estepa A, Griffiths G, Gjøen T.
Manuscript submitted for publication.

To further test the adjuvant effects of poly I:C, we made a subunit vaccine consisting of the recombinant VHSV glycoprotein G (rgpG). The protective effect of the rgpG compared to inactivated whole virus (iV) using chitosan-poly I:C nanoparticles as adjuvant was studied in zebrafish. Formulations containing free poly I:C + rgpG (pICrgpG) and free chitosan + rgpG (CSrgpG) were also compared. All vaccine formulations with poly I:C provided a

significant protection against VHSV; possibly through an early induction of an anti- viral state. Analysis of head kidney immune responses of all vaccine formulations 48 hours post-vaccination revealed that pICrgpG formulation conferred the highest induction of immune transcripts, upregulating TLR3, TLR8b1, TLR22, IFNs, and ISGs. All formulations containing poly I:C, upregulated Mx and ISG15 in zebrafish head kidney.

Paper V: Effects of dietary n-3 fatty acids on Toll-like receptor activation in primary leucocytes from Atlantic salmon (*Salmo salar*).

Arnemo M, Kavaliauskis A, Mira MB, Berge GM, Ruyter B, Gjøen T.

Manuscript submitted for publication.

The shortage of the n-3 fatty acids EPA and DHA on the international markets has led to increasing substitution of fish oil by plant oils in Atlantic salmon feed and thereby reducing the EPA and DHA content in the fish. EPA and DHA are known to modulate inflammation and immune responses in humans and experimental animals, but the minimum required dietary levels for securing fish health are unknown. Atlantic salmon were fed diets (growing from 50 to 400 gram) containing 0%, 1% or 2% EPA and DHA alone or in combination. Primary head kidney leucocytes were isolated and stimulated with TLR ligands to determine if EPA and DHA deficiency can affect expression of important immune transcripts and eicosanoid production. Several important genes related to a viral immune response did not vary between the groups. However, there was a tendency of the non-stimulated cells from high level EPA and DHA groups to express lower levels of IL-1β. These leucocytes were also more responsive to the TLR agonists, inducing higher expression levels of IL-1β and Mx after stimulation. The levels of PGE2 and LTB4 in serum and in media from stimulated leucocytes were lowest in both low and high EPA and DHA groups.

Discussion of results

The development of efficient and cheap vaccines against aquatic viruses is important for a sustainable aquaculture industry. Especially, there is a need for new fish vaccine adjuvants. TLRs are important receptors recognising pathogens, and TLR ligands are successfully used as adjuvants in human vaccines (Toussi and Massari, 2014, Steinhagen et al., 2011, Tomai and Vasilakos, 2012). To benefit the human adjuvant research, increasing the knowledge of fish TLR function and their ligands could provide valuable information leading to better adjuvant candidates in vaccines against viral diseases in fish.

I. TLRs and antiviral responses in fish

To provide overview of the immunostimulatory properties of TLR ligands in Atlantic salmon, we stimulated Atlantic salmon primary head kidney leucocytes with a range of classical TLR ligands (paper I). Although stimulatory effects in Atlantic salmon have been previously reported for certain TLR ligands (Jensen et al., 2002, Strandskog et al., 2008), no reports have compared a complete panel of classical ligands for all human TLRs (TLR1-9) on Atlantic salmon leucocytes.

In mammals, TLR3, TLR7, TLR8 and, TLR9 are recognised as the virus-sensing TLRs. Trough activation of the transcription factor family IRFs, they induce expression of type I IFNs resulting in an antiviral state that provides a crucial first-line defence against viral infections (Levy et al., 2001). IFNs lead to expression of ISGs that exert numerous antiviral effector functions (Schoggins and Rice, 2011). A group of well-known and important ISGs are the Mx proteins, which for example block intracellular viral transport and inhibit viral replication (Haller and Kochs, 2002, Lee and Vidal, 2002). The classical TLR ligands poly I:C (TLR3) and imiquimod (TLR7) are both widely used as mimics of viral infections in mammals and fish. As shown in paper I, these ligands upregulated IFN-α1, IFN-γ, and Mx expression in the Atlantic salmon leucocytes, suggesting induction of an antiviral state. This was confirmed in paper V as both ligands upregulated Mx in leucocytes from the "control group". Additionally, the important pro-inflammatory cytokine IL-1β was upregulated in the leucocytes upon exposure to poly I:C and imiquimod. Mx and IL-1b were also upregulated by poly I:C in the zebrafish leucocytes (paper III). There were some differences in how the zebrafish and Atlantic salmon

leucocytes were affected by poly I:C. However, both poly I:C concentration and duration of exposure were different in these studies. In addition, the whole head kidney leucocyte population was used from zebrafish in paper III, not only the adherent ones as in paper I and V. CpG ODNs are well-known to induce antiviral responses in fish and upregulation of Mx *in vivo* (Jørgensen et al., 2003, Strandskog et al., 2008, Carrington and Secombes, 2006, Jørgensen et al., 2001). Surprisingly, the CpG ODNs used in paper I and V did not upregulate Mx in Atlantic salmon head kidney leucocytes, but the IFN-α1 expression was increased.

In paper I we showed that the TLR expression in Atlantic salmon head kidney leucocytes was not affected by TLR ligand exposure. However, when analysing the expression of all known Atlantic salmon TLRs (at the time) in head kidney from ISAV infected fish, TLR3, TLR7, and TLR8a1 were highly upregulated. Atlantic salmon TLR3 was recently shown to also be upregulated during IPNV infection (Vidal et al., 2015). In addition to the virus-sensing TLRs, there are other possible sensors of viruses that may contribute to the induction of the antiviral state in the head kidney leucocytes in paper I and V. TLR13, TLR21, and TLR22 are also proposed as viral sensors in fish (Matsuo et al., 2008, Pietretti and Wiegertjes, 2013, Lee et al., 2014, Yeh et al., 2013, Poynter et al., 2015, Sahoo et al., 2014), and may contribute to the detection of poly I:C, imiquimod, CpG ODN, or viral infection. Also, other viral sensing PRRs, like the RLRs, were not investigated in paper I or V, but are probably also involved in sensing dsRNA in fish (see Figure 5) (Kawai and Akira, 2008, Poynter et al., 2015).

The zebrafish has emerged as a powerful model system for studying innate immune responses and infectious diseases (Novoa and Figueras, 2012). It is a simpler and cheaper model than Atlantic salmon and other aquaculture relevant species. In paper III, we tested the *in vivo* immunostimulatory effects of poly I:C in zebrafish. Six hours after poly I:C injection, Mx, TNF-α, and IFNϕ3 were upregulated, but the rest of the analysed genes were unchanged.

Because of the ability of poly I:C to induce strong antiviral responses, it has been widely explored as inhibitor of viral infection in fish (e.g. Atlantic salmon, zebrafish, rainbow trout, and Japanese flounder). Several reports demonstrate that *in vivo* prophylactic treatment with poly I:C can protect against or delay mortality after challenge with important aquatic viruses such as VHSV (Takami et al., 2010), IHNV (Kim et al., 2009,

Eaton, 1990), ISAV (Jensen et al., 2002), IPNV (Lockhart et al., 2004) and NNV (Oh et al., 2012). The combination of poly I:C with other TLR ligands can also have synergistic effects in increasing viral resistance, as demonstrated for CpG ODNs + poly I:C in Atlantic salmon (Strandskog et al., 2008, Strandskog et al., 2011) and LPS + poly I:C in zebrafish (Ruyra et al., 2014). In paper III, we tested if pre-treatment with poly I:C affected VHSV resistance. Consistent with several of the above mentioned studies, poly I:C delayed VHSV mortality, probably due to the induction of several genes involved in sensing and combating viral infection (TLR3, MDA-5, IRF-3, and Mx).

The immunostimulatory properties demonstrated in paper, I, III, and V, make poly I:C a promising candidate for a new adjuvant in fish virus vaccines. However, the protective effect of poly I:C combined with antigen needs to be quantified and compared to existing, commonly used vaccines.

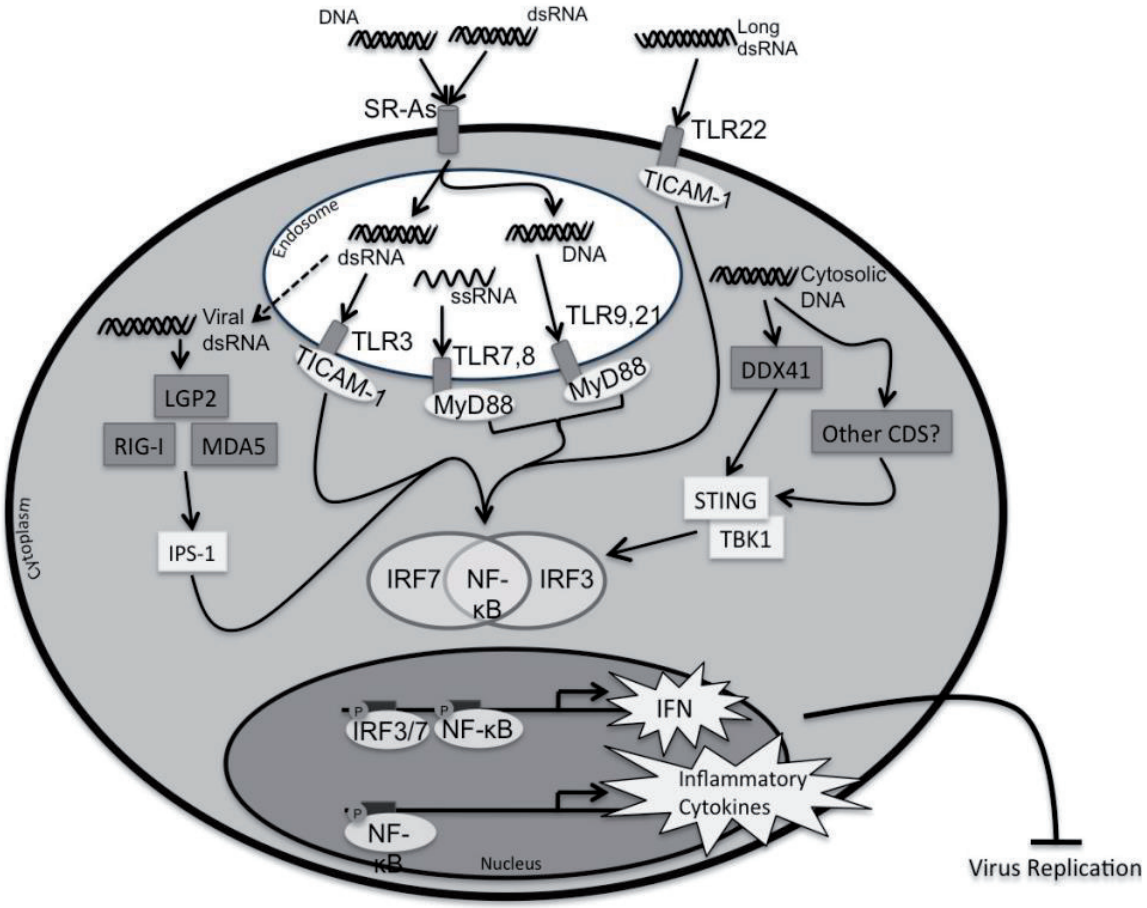

Figure 5: *Proposed fish nucleic acid PRR signalling pathways. Figure from (Poynter et al., 2015).*

II. Detecting TLR ligand specificity

Numerous papers discuss possible TLR ligand specificity in fish, however, the majority of the reports are based on gene expression analysis and there are very few functional studies. These analyses are often based on the assumption that TLR specificity is conserved between mammalian and fish TLRs, hence the use of one or two ligands when studying a specific TLR. Some of these receptors may have a wider specificity, and a complete range of ligands for mammalian TLRs should be used for initial screening. For the non-mammalian TLRs, ligands from fish pathogens could be used to detect putative natural ligands. It is also difficult to compare available reports since experimental design, pathogens, ligands, exposure time, dose of stimulant, and model system (e.g. cell lines, primary cells or *in vivo*) vary.

Although upregulation of a specific TLR or important immune genes following exposure to a TLR ligand, pathogen, or cytokine has been observed, this does not imply receptor-ligand binding. Many papers report no or only minor changes in TLR expression during pathogen, cytokine, or TLR ligand (e.g. in our paper I) (Langevin et al., 2012, Salazar et al., 2015, Strandskog et al., 2008, Brietzke et al., 2015, Lee et al., 2014, Palti et al., 2010a). The recruitment of cellular factors of innate immunity, rather than induced expression of the TLR itself, may be a key role for initiating the immune defence against a pathogen (Brietzke et al., 2015). In addition, several studies demonstrate upregulation of a TLR after few hours of stimulation with inflammatory cytokines (Lee et al., 2013, Skjæveland et al., 2009, Skjæveland et al., 2008). It seems that cytokines induce a more rapid upregulation of TLRs than the TLR ligands themselves, which suggests that this is an effect of kinetics and time of sampling. Cytokines are downstream effector molecules of the TLR signalling pathways, and upregulation of TLRs may be a secondary effect of the cytokine induction following recognition of the ligands or pathogen tested.

A direct correlation between ligand or pathogen binding and upregulation of a specific TLR is hard to demonstrate, but the upregulation can be considered an indication of a functional role for the TLR in recognition of the specific ligand or immune response to a certain pathogen.

The few available molecular tools for studying fish proteins (e.g. antibodies) limit fish immunology research; hence the extensive number of gene expression analyses by real-

time PCR. Although real-time PCR is specific and reproducible for mRNA quantification, correlation between mRNA and functional protein levels can vary due to several biological factors (e.g. translational efficiency and protein half-life) (Maier et al., 2009). The weakness of using real-time PCR as a single method therefore needs to be taken into consideration when interpreting gene expression data.

A common way of detecting ligand specificities for the mammalian TLRs has been *in vitro* reporter assays (Alexopoulou et al., 2001, Hemmi et al., 2002). In these assays, transcriptional activity can be measured in cells transfected with the TLR of interest and a genetic construct containing a reporter gene (e.g. luciferase) under the control of a promoter of interest (e.g. NF-κB) (Dellacasagrande, 2009). Signalling from all mammalian TLRs has been reported to ultimately activate NF-kB factors, and promoters for NF-κB are commonly used in reporter plasmids (Kawai and Akira, 2007).

Our goal was to establish a screening system for ligand specificities of fish TLRs. However, this turned out to be difficult, and in paper II we failed to activate rainbow trout TLR2 signalling when overexpressed in HEK-293 and salmon CHSE-214 cells. TLR2 transfected into HEK-293 quenched rather than increased the NF-κB activation in a ligand-independent fashion, as it has been shown for rainbow trout IRAK4 (Brietzke et al., 2014). Although fish TLRs and downstream factors of the TLR cascade seem evolutionary well conserved, there are pitfalls when expressing fish TLRs in mammalian cells (e.g. improper folding, lack of auxiliary factors or dimerization partner, and sub-optimal temperature). However, our efforts to establish the assay in a salmonid cell line did not reconstitute robust TLR signalling. Low transfection efficiency and lack of dimerization partners are possible explanations for lack of NF-κB activation in the fish cell line. To confirm TLR expression in HEK-293 and CHSE-214 when antibodies are not available, TLRs need to be tagged, which can interfere with signalling (Pang et al., 2009).

The literature reveals very few reports that successfully confirm ligand-dependent TLR activation of NF-κB or other relevant transcription factors in fish. Fugu TLR3 and TLR22 were successfully expressed in both HEK-293 and a rainbow trout cell lines, and relayed signals to activate IFN expression upon stimulation with dsRNA (Matsuo et al., 2008). The rainbow trout TLR5S activated NF-κB in HeLa cells; however, the extracellular domain was fused with the intracellular TIR domain of human TLR5 (Tsujita et al., 2004). Zebrafish TLR9 and TLR21 have been shown to activate NF-κB in HEK-293 cells, and

this activation was increased by co-transfection of Unc93B1, indicating the importance of accessory proteins in the reporter assays (Yeh et al., 2013). European common carp TLR2 could be expressed in HEK-293 and activated by PGN, LTA, and Pam3CSK4 when using p38-MAPK phosphorylation as a measure for responsiveness (Ribeiro et al., 2010). Although these few reports demonstrate activation of fish TLR in relevant reporter assays, from our own and others' experience, detection of ligand binding of fish TLRs in these assays appear very challenging.

The use of knockout mice is another way of investigating the role of TLRs during infection and possible ligand specificity in mammalian TLR research (Akira, 2000). A recent report demonstrated that TLR3 and MDA-5 knockdowns in Japanese flounder reduced poly I:C mediated antiviral activity (Zhou et al., 2014). In addition, zebrafish embryo TLR2 and TLR5 knockdowns displayed inhibited Pam3CK4 and flagellin responses, respectively (Yang et al., 2015). The reports demonstrate the importance of these PRRs in immune responses induced by the ligands tested. Increased number of tools for laboratory methods in fish (e.g. mutants and antibodies) will enhance the future functional studies of fish TLRs, their ligands, and signalling pathways.

III. Poly I:C as vaccine adjuvant

Adjuvants can be divided into two groups, delivery systems (e.g. emulsions) and immunostimulatory components (e.g. PAMPs), and often a combination of the two is required. The principle of using PAMPs like poly I:C or other TLR ligands as adjuvants is not new to mammalian vaccine research and there are several reviews of their effects available (Hafner et al., 2013, Steinhagen et al., 2011, Toussi and Massari, 2014, Tomai and Vasilakos, 2012, Martins et al., 2015).

Poly I:C stimulation in mammals induces a strong type I and III IFN and Th1 cytokine response. Poly I:C is a strong Th1-inducing adjuvant that makes it attractive for use in vaccines against viruses and other intracellular pathogens, but the responses vary depending on cell types, receptors, and cytokines surrounding the administration site (Martins et al., 2015).

Poly I:C has successfully been tested as prophylactic treatment against several important aquatic viruses. However, there are only few reports considering the adjuvant effects in a fish vaccine. In this thesis, the adjuvant effects of poly I:C in two different VHSV vaccines

were tested; one inactivated whole virus vaccine (Paper III) and one subunit vaccine (Paper IV). We established the previously described zebrafish cold-water system (Novoa et al., 2006) to test our vaccine formulations.

There is no doubt that poly I:C is a strong and efficient antiviral immunostimulant in both humans and fish. However, experience from experimental animals and clinical trials shows that it can trigger autoimmunity and toxic effects when used systemically and in high dosages. In addition, free poly I:C is rapidly degraded by serum nucleases, thus effectively reducing the concentration (De Clercq, 1979). More stable derivatives of poly I:C (e.g. poly I:C12U and poly ICLC) have been developed in attempts to modulate toxicity, immunogenicity and half-life. A commonly used strategy to balance immunogenicity and safety is to use a delivery system to optimise activation of antigen presenting cells (without excessive systemic effects) at the lowest dosage possible (Hafner et al., 2013, Martins et al., 2015). Multiple delivery systems exist (e.g. liposomes, emulsions, alginate, chitosan, and PLGA) for use in vaccines (Reed et al., 2013, Hafner et al., 2013). In such a delivery system, the antigen and/or immunostimulatory adjuvant (e.g. poly I:C) is encapsulated in a nano- or microparticle made up of a biodegradable polymer that allows a slower release of the antigen and/or immunostimulatory adjuvants (Griffiths et al., 2010). Several reports have demonstrated that the effects of immunostimulants can be enhanced by macrophage targeting, using micro- or nanoparticles (Singh et al., 2007). Therefore we tested chitosan-encapsulated poly I:C in our VHSV vaccines.

In paper III, chitosan-encapsulated poly I:C was co-injected with inactivated whole VHSV. The expression profiles of antiviral TLRs, cytokines, and antiviral proteins in zebrafish head kidney suggested strong induction of an antiviral state 48 hours post-vaccination. The fish were challenged 31 days later with live VHSV virus (and re-challenged 20 days later). The vaccine provided a significant protection against VHSV-induced mortality.

In paper IV, we tested vaccines containing the VHSV glycoprotein G with either poly I:C or chitosan, or a combination of both. The average size of the chitosan-encapsulated poly I:C particles was 368 nanometres, thus the name nanoparticles. The early immune responses (48 hours post-vaccination) to vaccination was strongest for the vaccine group injected with glycoprotein G and free poly I:C. This formulation increased expression of antiviral sensors (TLR3, TLR8a1, and TLR22) and IFNs. This is probably due to increased free poly I:C leaving the site of injection by the blood stream, whereas the chitosan-

encapsulated poly I:C formulation possibly has a depot effect that promotes sustained release of poly I:C. All formulations containing poly I:C increased expression of Mx and ISG15, and conferred significant protection against VHSV-induced mortality. Encapsulating poly I:C in chitosan clearly delayed mortalities compared to free poly I:C, although the final mortality was similar in both groups. Poly I:C in combination with CpG ODNs has previously been reported to increase the immunogenicity and protection of a low dose inactivated SAV vaccine in Atlantic salmon (Thim et al., 2012, Thim et al., 2014). These studies, combined with results from our vaccination trials, suggest that poly I:C is a promising adjuvant candidate for future vaccine formulations containing antigens with low immunogenicity. Further, our results also suggest that zebrafish is a valuable model for aquaculture-relevant vaccination studies. However, we need to take into account that challenge by injection is different from the natural route of viral infection. Future studies need to focus on possible adverse effects in the fish upon use of TLR ligands as adjuvants.

Although the injection vaccines have several advantages (e.g. direct delivery of antigen, low antigen dose needed, concentrated antigen, protected in highly purified format, and refrigerator storage), the method is stressful for the animals, can cause tissue injury, are labour intensive, cannot be repeated multiple times during the production cycle (for economic reasons), and requires anaesthesia. The ideal vaccine should induce long lasting protection starting at an early age and protect the fish throughout the production cycle. Compared to injections, oral administration is non-invasive, fish do not need to be handled, can be given to fish at any age, will reduce labour cost, and can be repeated (i.e. booster dose). However, oral vaccines have proven difficult to develop, and challenges such as low immunogenicity, antigen formulation, and storage in high temperature and humidity need to be solved. Mucosal vaccines are beginning to appear for human use, for which chitosan seems to be a promising delivery system (Amidi et al., 2010, Smith et al., 2014, van der Lubben et al., 2001). Although the vaccine formulation containing chitosan and glycoprotein G without poly I:C proved not to be protective in paper IV, several other reports have demonstrated chitosan as a potential carrier for antigens delivered via the oral route in fish (Rajesh Kumar et al., 2008, Tian et al., 2008, Rivas-Aravena et al., 2015). Since the knowledge of mucosal immunity in fish is increasing (Beck and Peatman, 2015), future studies should aim to investigate whether encapsulated TLR ligands (e.g. in chitosan) could be used in oral vaccines.

IV. Dietary influence on immune responses

The composition of fatty acids in membrane phospholipids of mammalian immune cells strongly correlate with the profile of dietary lipids (Calder, 2013). Membrane phospholipids are a part of the microenvironment for leucocyte transmembrane signalling receptors and are important substrates for enzymes of eicosanoid metabolism. Due to the reduced EPA and DHA content in Atlantic salmon feed, and the subsequent reduction of these n-3 fatty acids in the animals, it is important to investigate if the decreased EPA and DHA levels can affect immune function and further influence disease resistance and vaccine response. Therefore, in paper V, we investigated the effects of EPA and DHA on primary head kidney leucocytes from Atlantic salmon. This project is a part of a bigger project ("Minimum requirements for omega-3 fatty acids in modern production of Atlantic salmon") in which other partners are investigating the effects of EPA and DHA on growth and quality.

The head kidney is the principal immune organ for phagocytosis and antigen processing in fish (Rauta et al., 2012); hence it is rich in immune cells. Our analysis of the head kidney confirmed that the EPA and DHA levels in this tissue correlated with the dietary levels of these fatty acids. This correlation is widely recognised in mammals (Calder, 2013), but has also previously been demonstrated in several fish species (Bell et al., 1993, Bell et al., 1992, Bell et al., 1996, Bell et al., 1998, Ganga et al., 2005). In order to investigate the role of altered fatty acid composition in head kidney on immune function, we isolated head kidney leucocytes from Atlantic salmon that had been fed various levels of EPA and DHA. The leucocytes were stimulated with TLR ligands (as in paper I) or infected with viruses (ISAV and IPNV) and analysed for expression of important immune genes. The majority of the genes analysed in non-stimulated leucocytes were not affected by dietary EPA and DHA, but IL-1β was expressed at lower levels in the high EPA and DHA dietary groups, thus indicating anti-inflammatory effects (like widely recognised in mammals (Calder, 2013)). However, after stimulation with TLR ligands, leucocytes from fish that had been fed low EPA and DHA were unable to upregulate the important immune transcripts IL-1β and Mx1, which indicates that the absence of dietary EPA and DHA may have immunosuppressive effects. The reduced response to the mimics of bacterial and viral infection could potentially make these fish more prone to infections. In addition, vaccine effects in these fish can potentially be reduced (e.g. if poly I:C is used as adjuvant).

Even though replacement of fish oil by vegetable oil is an important topic, there are few studies focussing on its effect on disease resistance and vaccine effects (most studies focus on the effects on growth and quality). There are a few studies on Atlantic salmon, where partial or full replacement of fish oil by vegetable oils did not appear to affect vaccination efficacy (Montero and Izquierdo, 2010). There is a disagreement in the existing literature about n-3 fatty acids and infectious disease resistance in mammals (Anderson and Fritsche, 2002) and fish. Some *in vitro* studies indicate that low n-3 fatty acids decrease disease resistance in fish (decreased phagocytosis, bacterial clearance, and respiratory burst) (Kiron et al., 1995, Sheldon and Blazer, 1991, Montero et al., 2008, Montero et al., 2003), while others report no effect of low EPA and DHA on leucocyte activity (Seierstad et al., 2009, Montero et al., 2003, Gjøen et al., 2004). *In vivo* experiments have also produced different outcomes, in which dietary vegetable oils have been demonstrated to decrease, increase, or show no effects on mortality after bacterial challenge (Waagbø, 1994, Thompson et al., 1996, Gjøen et al., 2004, Montero and Izquierdo, 2010). Since the study design and feed content varies highly among available reports, it is difficult to explain the contradictory results. Our data indicated that higher tissue levels of EPA and DHA confers increased sensitivity in leucocytes stimulated with TLR agonists. This supports the previous findings that demonstrate that n-3 fatty acids are important for diseases resistance. However, further research is required to understand the role of dietary fatty acids on resistance to pathogens and immunostimulants in fish.

Main conclusions

- Head kidney and spleen are the main TLR expressing tissues in Atlantic salmon. Several TLR ligands induced expression of inflammatory cytokines in salmon head kidney leucocytes. The TLR3 ligand poly I:C, induced expression of antiviral transcripts in primary head kidney leucocytes. TLR3, TLR7, and TLR8a1 were induced *in vivo* after viral infection.
- Although rainbow trout TLR2 domain structure and ligand-binding sites seemed to be evolutionarily conserved, classical TLR2 ligands failed to activate TLR2 signalling *in vitro*.
- Poly I:C induced expression of antiviral transcripts in zebrafish both *in vitro* and *in vivo,* and delayed mortality after VHSV challenge.
- Poly I:C formulated with chitosan co-injected with inactivated VHSV provided protection against subsequent VHSV infection in zebrafish. Also, poly I:C alone or combined with chitosan co-injected with recombinant VHSV glycoprotein G provided protection against VHSV.
- Although few genes were affected by EPA and DHA in the non-stimulated leucocytes, the ability of these leucocytes to respond to TLR ligand stimuli was reduced with low dietary and head kidney levels of EPA and DHA, thus indicating the importance of n-3 fatty acids in resistance to infection and response to vaccines.

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Scientific paper I-V

Paper I: Effects of TLR agonists and viral infection on cytokine and TLR expression in Atlantic salmon (*Salmo salar*).
Arnemo M, Kavaliauskis A, Gjøen T. *Developmental and Comparative Immunology (2014). 46, 139-145.*

Paper II: Structurally diverse genes encode Tlr2 in rainbow trout: The conserved receptor cannot be stimulated by classical ligands to activate NF-kappaB in vitro.
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Paper III: Use of poly I:C stabilised with chitosan as a vaccine-adjuvant against Viral Haemorrhagic Septicaemia Virus infection in zebrafish.
Kavaliauskis A, **Arnemo M**, Kim SH, Ulanova L, Speth M, Novoa B, Dios S, Evensen Ø, Griffiths GW, Gjøen T. *Zebrafish (2015). 12, 421-431.*

Paper IV: Chitosan-poly I:C nanoparticles: a novel adjuvant in aquaculture vaccines. A study of particle bio- distribution and immune response in zebrafish (*Danio rerio*).
Kavaliauskis A, **Arnemo M**, Speth MT, Lagos Rojas LX, Rishovd AL, Estepa A, Griffiths G, Gjøen T. *Manuscript submitted for publication.*

Paper V: Effects of dietary n-3 fatty acids on Toll-like receptor activation in primary leucocytes from Atlantic salmon (*Salmo salar*).
Arnemo M, Kavaliauskis A, Mira MB, Berge GM, Ruyter B, Gjøen T. *Manuscript submitted for publication.*